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RESEARCH

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CIBA FOUNDATION SYMPOSIUM

ON

LEUKÆMIA RESEARCH

Editors for the Ciba Foundation

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PREFACE

Sir Henry Dale, Professor L. J. Witts and Dr. F. H. K. Green made the suggestion early in 1953 that it would be valuable to have an opportunity to discuss informally on an international level the present status of research in leukæmia and the directions in which further research might most profitably be pursued. They put forward this proposal on behalf of the (European) Scientific Advisory Committee of the Lady Tata Memorial Trust, a charitable Trust founded by the late Sir Dorabji Jamsetji Tata of Bombay in memory of his wife, which has become world-famous in the last twenty-one years for its support of research on diseases of the blood, and more especially leukæmia. The Director of the Ciba Foundation very willingly agreed to organize such a symposium on the lines of the previous conferences held at the Foundation, and he is particularly indebted to Dr. Green and Professor Witts for much essential advice in its arrangement, and to Sir Henry Dale for his whole-hearted support both of the project and of the actual meeting in November, 1953.

To those to whom this book serves as an introduction to the activities of the Ciba Foundation it should be explained that it is an international centre, which is established as an educational and scientific charity under the laws of England. It owes its inception and support to its founder, Ciba Ltd. of Switzerland, but is administered independently and exclusively by its distinguished British Trustees.

The Foundation provides accommodation for scientific workers who visit London from abroad, organizes and holds international symposia, conducts (in conjunction with the Institut National d'Hygiène) a post-graduate medical exchange scheme between England and France, arranges informal meetings for discussions, awards an annual lectureship, assists international congresses and other scientific societies,

is building up a library service in special fields, and generally endeavours to give aid in all such matters as may promote international co-operation in scientific research.

Leading research workers from different countries and in different disciplines are invited to attend the symposia or colloquia. The size of the groups is, however, very strictly limited in order to obtain a free conversational manner of discussion—*although the basic timetable of the programme* is strictly observed. The smallness of the groups necessarily means the exclusion of many other workers active and interested in the subjects discussed, and therefore the proceedings of these conferences are published and made available throughout the world.

It is hoped that the papers and discussions in this book will prove not only informative and stimulating, but will also give to readers a sense of participation in an informal and friendly occasion.

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M. Bessis	National Blood Transfusion Centre, Paris
Willemina M. de Bruyn	Netherlands Cancer Institute, Amsterdam
J. H. Burchenal	Memorial Center for Cancer and Allied Diseases, New York
J. Craigie	Imperial Cancer Research Fund, London
Sir Henry Dale	Wellcome Trust, London
J. F. Danielli	Dept. of Zoology, King's College, University of London
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Astrid Fagraeus	State Bacteriological Lab., Stockholm
J. Furth	Children's Cancer Research Foundation, Boston
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F H K. Green	Medical Research Council, and Lady Tata Memorial Trust, London
L. Gross	Veterans Administration Hospital, Bronx, and Sloan-Kettering Institute, New York
A. Haddow	Chester Beatty Research Institute, London
P B Hansen	Radiological Clinic, Aarhus, Denmark
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L. O. Jacobson	Argonne Cancer Research Hospital, University of Chicago

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L. W. LAW	National Cancer Institute, Bethesda, Md.
E. LORENZ (deceased)	Lab. of Biophysics, National Cancer Institute, Bethesda, Md
S. MOESCHLIN	Dept. of Medicine, University Clinic, Zurich
R. H. MOLE	Radiobiological Research Unit, Harwell
G. NEGRONI	Imperial Cancer Research Fund, London; and Istituto di Patologia Medica, University of Naples
C. OBERLING	Cancer Research Institute, University of Paris
EDITH PATERSON	Christie Hospital and Holt Radium Institute, Manchester
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CHAIRMAN'S OPENING REMARKS

L. J. WITTS

THE impulses which drive men to engage in research are doubtless numerous and complex, but in opening this symposium on leukæmia research I shall mention only two. The first is the spirit of curiosity, a curiosity both intellectual and æsthetic, and the second is the desire to relieve human suffering. The study of diseases of the blood is of intense interest in its own right. The physical appearance of the blood cells and their movements have always had a fascination, whether seen through the primitive instruments of Leeuwenhoek, Malpighi and Swammerdam in the seventeenth century or through the electron microscope today. By staining the blood cells we can produce pictures of great beauty, and at least one American hæmatologist has a hand-painted neck tie which is an accurate delineation of a marrow smear. Equally subtle techniques are available for measuring the rate of formation and of destruction of the blood cells and for studying many aspects of their life history in health and disease.

Leukæmia is an obvious subject for inquiry because in leukæmia bizarre changes occur in both the appearance and the behaviour of the white blood cells. We are made to wonder at something we had perhaps too easily taken for granted, the homeostatic mechanism which normally maintains within well defined limits not merely the total number of white cells but the several members of the white-cell series in the peripheral blood, and which silently takes care of their entry into and their departure from the circulating blood stream. This homeostatic mechanism responds to infections, stresses and other factors which alter the complexion of the

white blood count by mechanisms which are still imperfectly understood and whose disturbance in leukæmia may therefore form a point of departure for physiological studies.

It is rarely possible, however, for the medical researcher not to be aware on occasion of the human or emotional aspects of the problems which he is studying, and few diseases are more tragic than leukæmia or more inexplicable in terms of human suffering. A generation ago it might have been regarded as a relatively rare and unimportant disease, but this is no longer the case. Leukæmia now accounts for more deaths in childhood than all the common acute infections put together, and it forms a significant portion of malignant disease in later life. In animal husbandry likewise it takes an important toll from our herds and flocks.

This Ciba Foundation type of conference has established the value of bringing together workers from different nations and different disciplines in a small and informal conference in which there is time and room for the cross-fertilization of ideas, but we also had another purpose in mind in this particular symposium. A number of foundations are now specifically interested in the problem of leukæmia and our meeting this week grew out of a discussion between the secretaries of the Ciba Foundation and the Lady Tata Memorial Trust. We who are on the Scientific Committee of the Lady Tata Memorial Trust are proud of some of the work which it has sustained or to which it has contributed during the last twenty years. It will not be invidious for me to refer to the pioneer studies of Professor Engelbreth-Holm in Copenhagen, of Jacob Furth at Cornell and of Professor Roussy in Paris, and to say what a pleasure it is to have Engelbreth-Holm and Jacob Furth with us here today. Steady progress has been made in the understanding of leukæmia and there has been some small but measurable improvement in methods of treatment. Our programme shows that the difficulties of research in leukæmia have not dissuaded first-class workers or prevented significant discoveries.

We felt there was a striking contrast between the high level of these researches and the lack of inspiration and knowledge revealed in some of the applications we have received for scholarships and grants. Project research is always open to the criticism that it encourages the irresponsible or mercenary applicant, but we have to accept the fact that project research is likely to be with us for some time to come and that considerable funds are already earmarked for research on leukæmia. Moreover, one feels less diffident about this than about some other forms of project research, for leukæmia appears to offer a promising salient in the fight against malignant disease. In addition it brings together in an unusually intimate way those who are working in the basic scientific fields and those who may more properly be called clinical workers, because of the fact that man himself is often the most suitable animal for experimental work on leukæmia.

This symposium should therefore be of great value to those of us who are members of research committees and may here discover those laboratories and foci of activity to which research students in leukæmia may most profitably be directed. Similarly, the publication which we hope to see emerge from this symposium will be of great value to younger workers or entrants into this field who may thereby learn which areas of knowledge are already fully developed, which problems are clearly defined and which techniques at the moment appear to be most promising As they are young we may also hope that the newcomers will see the openings which those of us who are older have overlooked

Most of us—I know not all of us, but most of us—feel that leukæmia is part of the total problem of cancer and for this reason many of the cytological and physicochemical methods of study are common to the whole field. I hope you will find that these various approaches are well covered in our programme If there is a hiatus, and doubtless it will be filled during the course of the discussion, it is the question of the nutritional requirements of the leukæmic cell and the

possibility of influencing the disease by nutritional means, of which some hints have appeared from time to time in the literature. We have allotted relatively little time to the clinical aspects of leukæmia, with the exception of the newer forms of treatment. The incidence of the disease in Western Europe and North America, its classification, morbid anatomy and natural history, and the effects of established treatments have now been worked out with considerable accuracy. Here too, however, there remain certain areas which still require exploration. The first is the geographical pathology of the disease. For example, it is said that lymphosarcoma is unduly frequent in the natives of South Africa, and this is the type of finding that would be worth confirmation and explanation. The second clinical or demographic problem lies in the evidence that leukæmia is increasing in frequency in Western Europe and America and changing in the distribution of the various types, and this is an aspect of the disease that might well be worthy of sustained and accurate observation.

ON LEUKÆMIA RESEARCH

J. ENGELBRETH-HOLM

In this paper I shall endeavour to outline some topics within leukæmia research, clinical as well as experimental, which in my opinion should be investigated, including topics in which interesting observations have already been made but which have not, however, been further examined.

At the present time the classification of the leukæmias is rather obscure, even more so than ten to twenty years ago, and the relation between leukæmias in man and animals has become more vague. In the myeloid leukæmias difficulties arise as to which attitude to adopt towards myeloid metaplasia, myelosclerosis, and non-leukæmic myeloid splenomegaly, polycythæmias and leukæmic erythroblastosis. In animals where myeloid leukæmia has been investigated, conditions are less obscure than in man In the former the tumorous nature of myeloid leukæmias cannot be denied, whereas in man the question may be whether one tumour group should be recognized, including the classical myelogenous leukæmia, particularly the myeloblast leukæmias and chloromas (analogous to myeloid leukæmia in fowl and mouse), and another non-tumour group, including chronic myelogenous leukæmias, polycythæmias, myosclerosis, etc. It is very interesting to notice that myelogenous leukæmias, found in fowl, are excited by the same virus as that which excites erythroblastosis and which in certain slowly progressing cases brings about myelosclerosis. However, it is questionable whether one is justified to draw an analogy between the above diseases and those with similar names in man.

Recently, Dameshek (1951) put forward the hypothesis that all the conditions mentioned above were different reactions to a myelostimulatory principle. The theory is a pleasing one, but if all these diseases are to be considered as

an entity, it is almost impossible to explain the nature of the pathogenetic processes. It is beyond doubt that true leukæmias in fowl and mouse are of tumour nature whereas myosclerosis, myeloid metaplasia, and non-leukæmic myeloid splenomegaly in man are definitely not tumorous.

In this respect we are confronted with a peculiar feature. It is generally agreed that true leukæmias should be considered as malignant tumours. But what are the corresponding benign tumours? It has often occurred to me that in this respect something is amiss in our classification. I am aware of no other tissue which may become the site of malignant tumours without the capacity of developing benign tumours. Might not some or more of these leukæmoid conditions be considered as the benign counterparts of the malignant leukæmia?

When we come to lymphogenous leukæmia we find difficulties of a different kind. This type of leukæmia has been mainly investigated in fowl, mouse, and cattle, as well as in man. Whereas the mouse leukæmias offer no obstacles as to their classification—all of them being obviously tumorous—there is doubt as to the disease in fowl and man. Certain chronic lymphocyte forms deviate somewhat from the more acute forms. So far the chronic form in fowl has not been proved to be caused by a virus, as opposed to the acute form (and the neurolymphomatosis, a disease specific to fowl); and Bethell (1942) and Wiseman (1942) voiced the possibility that chronic lymphocytic leukæmia is different from the tumorous leukæmias and is possibly of a metabolic nature.

It is still uncertain where to place cattle leukæmia. In some cases it has been defined as a metabolic disease, but its origin is still being discussed.

It is also being discussed whether the chronic, slowly progressing lymphogenous leukæmia must be considered as deviating basically from the acute lymphosarcomatous conditions, or whether it should be classified as a more highly differentiated, relatively benign tumour of the lymphatic tissue.

Now, the most important problem is to determine which course to follow in order to elucidate further the classification of the leukæmia diseases. It is hardly practicable to proceed by purely morphological and clinical studies, but a quantitative morphology, combined with measurements of nuclei and cell sizes (as attempted by, e.g., Heiberg (1930), and Engelbreth-Holm (1933)) might help to elucidate our present rather uncertain classification.

Another sphere in which continued and intensified investigations are desirable is the ætiology and interrelation of the different causative agents of the leukæmias. Much knowledge has already been gathered in this respect. It is a fact that tumour-exciting influences like hydrocarbons and radioactivity may be concurrent causes for the development of leukæmia, and it is not unreasonable to include inheritance as a factor. But other conditions which may have a decisive influence on the development of leukæmia must also be taken into consideration. I am thinking in particular of the very interesting finding of Saxton, Boon and Furth (1944) that underfeeding of mice reduced the incidence of spontaneous leukæmia from 65 per cent to 10 per cent. I wonder why this observation failed to lead to more extensive qualitative studies on the importance of food in the development of leukæmia. However, some examinations have been performed. Lysine and tryptophan deficiency, for instance, seem to have no inhibiting effect on the development of leukæmia in mice, whereas cystine deficiency in the food reduces the leukæmia incidence (White, White and Mider, 1948). Such conditions are in my opinion of the utmost importance and should be investigated much more thoroughly. Several similar investigations have been performed on other tumours, but it holds true here as in the other types of malignant tumours that no generalization is justifiable among the various tumours. However, the restriction of the caloric intake of animals seems to reduce the incidence of many spontaneous tumours besides leukæmia.

In this connection the investigations into the various

hormonal influences on the development of leukæmia should be mentioned. In several strains of mice there is a definite difference in incidence in males and females. Here it is noteworthy that in man chronic lymphogenous leukæmia occurs most frequently in elderly men. Œstrogens play an important role in causing leukæmia in certain strains, whereas testosterone is inhibitory. Cortisone too has a definite inhibitory effect on the development of lymphogenous leukæmia in mice (Kaplan *et al.*, 1951; Woolley and Peters, 1953).

The various hormonal influences during the development of leukæmia deserve further investigation, although, as stated by Furth (1952), "hormones appear to be modifiers rather than direct inciting agents." It goes without saying that it would be of the greatest importance to have the actual ætiology of leukæmia disentangled, to find out whether a single ætiological agent is concerned, or whether it is a combination of various factors, as is the case in several other tumours.

To assume a single ætiological agent is for all practical purposes almost identical to the assumption of a virus as the causative factor in leukæmia in man and mammals, as has been found in fowl It should, however, be remembered that a virus need by no means be the only cause of leukæmia, merely one of several co-operating factors which finally result in the development of the disease. Such conditions are known, e g in breast carcinoma in mice the milk factor is apparently one of several necessary factors

In 1951 Gross reported experiments with injections of extracts of leukæmic cells or of normal embryos of the leukæmic strain AK into C3H mice less than ten hours old. Many of the treated C3H mice developed leukæmia later in life, as opposed to the non-treated C3H mice. These very interesting experiments, indicating a virus as the cause of the leukæmia in AK mice, have been subject to criticism, but I shall leave them for the present time, since we shall have the opportunity of discussing those conditions tomorrow when Dr. Gross will talk about his recent experiments.

It is not surprising that I take a keen interest in these experiments when it is borne in mind how I thought I had transferred AK leukæmia with cell-free material, in experiments which later failed to be repeated by both myself and MacDowell. However, I never succeeded in finding the "flaw" in the first series of eight experiments (Engelbreth-Holm, 1948).

Experiments on this and similar lines are urgent and must be anticipated with the highest interest.

Experimental therapy of leukæmia will not be discussed here, since we shall in the course of our symposium hear several papers on chemotherapy in man and animals. I shall confine myself to directing your attention to the very interesting experiments in recent years on the infection of tumour-bearing animals with various viruses, particularly neurotropic ones, which will kill tumour cells when growing in them (e.g. Moore, 1951). Most of the experiments were performed on tumours other than leukæmias. Turner *et al.* (1948) reported, however, that vaccinia virus has an inhibitory effect on AK leukæmia in the mouse. Quite recently Southam and Epstein (1953) examined the effect of several viruses on AK leukæmia. They found that the Russian spring-summer encephalitis virus inhibited leucocytosis and organ infiltration. The survival time, however, was not affected. Five other viruses showed slight anti-leukæmic action whereas nine virus strains were ineffective

Further experiments on this line might be of interest, although experimental [illegible] tumour cells and the corresponding normal cells Almost all therapy of leukæmias and other tumours (apart from surgical therapy purporting to remove tumour tissue from the organism) attempts to kill tumour cells, whereas a rational therapy should aim at restoring the abnormal cells to normal conditions.

This leads directly into the last but not least important field in which I think future research should be conducted.

Our knowledge of the factors regulating the normal production of the cells, which in leukæmia proliferate in a tumorous way, is rather scanty, and we know little or nothing about the factors which are necessary for the normal differentiation of blood cells and which are absent or defective in the leukæmias.

One single factor—vitamin B_{12}—is known from research on pernicious anæmia to be fundamental to normal differentiation, *not merely of the erythrocytes, but of all marrow cells.* There may be other, hitherto unknown factors, which are lacking in leukæmias.

In 1940, Nettleship reported that extracts of marrow and leucocytes are inducers of leucocytosis, and in the same year Menkin published his first paper on a factor liberated from injured cells in exudates capable of inducing a leucocytosis in animals. He further developed this observation (Menkin, 1948), and it is of particular interest to our present issue to notice that he says: "it seems as if repeated subcutaneous injections of the leucocytosis-promoting factor of inflammatory exudates, when repeatedly administered to experimental leukæmic mice, induce a shift in the differential leucocytic formula with a concomitant rise in the percentage of mature polymorphonuclear leucocytes occurring in the circulating blood."

If this hypothesis were substantiated it would be highly important. Any case whatever of increased differentiation of tumour cells requires our urgent attention since the most obvious feature in malignant tumours seems to be a defective or even lost differentiation.

Cases of splenogenic marrow inhibition may also be examples of absent differentiation factors. Johansen (1950) put forward the hypothesis that splenic anæmia and other instances of splenic marrow inhibition might possibly be explained by assuming a factor necessary for the normal development of the different cellular elements of the marrow, this factor being absorbed by the reticulo-endothelial elements of the enlarged spleen and thereby depriving the marrow of the factor. In tissue-culture experiments he actually found

some evidence of the presence of this otherwise unknown factor.

Every single indication of factors which seem to be of importance in the production or differentiation of bone-marrow cells deserves careful investigation in animal experiments, and as far as possible in tissue cultures. In the study of white blood cells the tissue-culture technique has hitherto been of only minor importance. It should be possible to develop more refined and reliable techniques by which to investigate these cells as well as their normal or abnormal precursors. Factors necessary for differentiation, and the effect of amino-acids and other food constituents could be evaluated in far more detail than in animal experiments.

It seems essential to increase our knowledge as to the normal development of the precursors of the white blood cells. Without achieving this the danger is that we shall have only small success in interfering rationally with the diseased cells of the various leukæmias

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THERAPEUTIC CORRELATION BETWEEN MOUSE AND HUMAN LEUKÆMIA*

JOSEPH H. BURCHENAL

This afternoon I would like to discuss with you the correlation between mouse and human leukæmia. Furth, Ferris and Reznikoff (1) have demonstrated that almost all forms of human leukæmia may be mimicked by mouse leukæmia, with the possible exception of the small cell chronic lymphocytic leukæmia. Today, however, I would like to consider with you the biochemical correlation between human and mouse leukæmias in their response to treatment. Those of you who are clinicians do not need to be reminded, I am sure, of the great differences in regard to response to chemotherapeutic agents which can occur between two patients with acute leukæmia whose disease morphologically appears to be identical. Similarly, mouse leukæmias are not all identical biochemically and their response to differing agents varies as much as in the human leukæmias. The responses of human leukæmias to the conventional agents are well known; and similarly the effect that may be produced on most of the well-known transplantable strains of leukæmia by conventional agents has been studied. Today I propose to consider some nine physical and chemical agents which are useful in the treatment of leukæmia in man and show the correlation between human and mouse leukæmias in regard to these agents.

In discussing the effects of physical and chemical agents which inhibit leukæmias in mice and man, one must rely to a large extent in mice on the data gained from various lines of

*From the Chemotherapy Service, Memorial Center for Cancer and Allied Diseases, the Division of Experimental Chemotherapy, Sloan-Kettering Institute, and Sloan-Kettering Division of Cornell University Medical College, New York, New York.

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[Discussion of this paper was postponed until after the paper by *Dr. Israels*.—*Ed.*]

The important point is to have some way of picking up agents which will be useful in the patient. We are not interested in curing mice. If we were, we would not have given them the leukæmia in the first place.

In the correlation between cell type and response to chemical agents, the generalization can be made that the chronic myelocytic types of leukæmia showing many promyelocytes, myelocytes, metamyelocytes, etc., in both mouse and human leukæmias will respond to one group of agents; whereas the more acute stem-cell leukæmias will respond to another group. Beyond this, however, the classification according to morphology breaks down. In both patients and in mice two acute leukæmias having similar clinical signs, indistinguishable morphologically, and causing

[illegible]

that in the future, human laukæmias, particularly of the acute type, will be classified more and more according to their biochemical characteristics, as shown by their response to chemotherapeutic agents, and less by their morphological characteristics, on which it is often hard to get three morphological hæmatologists to agree.

In man, X-ray is a valuable form of therapy in the chronic myelocytic and chronic lymphocytic leukæmias. It has generally been thought that although it increased the useful and comfortable life of the patient, it did not increase his actual survival, but recent figures from Osgood (3) seem to indicate that regularly spaced titrated doses of irradiation may cause actual increase in survival time as well. In chronic myelocytic leukæmia, radiation therapy either by localized X-ray over the spleen, total body spray, or by the oral or intravenous use of radioactive phosphorus remains the most thoroughly studied and most widely employed form of therapy, but it obviously can be employed only in co-operation with qualified radiologists

In mice, Furth and Kabakjian (4) showed a definite increase in survival time with Line A35 transplanted leukæmia

transplanted leukæmia. To quote from Kirschbaum's excellent review of rodent leukæmia (2):

> "It must be recognized that the transplanted leukemia is quite different from either the induced or spontaneous disease, in that the cells of the leukemic animal did not undergo their malignant transformation in the host, but are the progeny of cells which were malignant when introduced.
>
> ". . . Although delaying or inhibiting the development of transplanted leukemia by instituting treatment within one to four days after transplantation may have significance, and although drugs demonstrating this effect have usually had the greatest effect upon human lesions, it must be recognized that inhibiting transplanted mouse leukemia is a far cry from successfully treating spontaneous mouse leukemia, not to mention human leukemia."

While I agree thoroughly with the reviewer that there is a tremendous difference between transplanted and spontaneous or induced leukæmia, still I would like in this short discussion to show that even in the transplanted leukæmia there is good correlation with the human disease in respect to response to therapeutic agents. The reason for using transplanted leukæmia is that it lends itself much better to large-scale screening studies; reproducible results can be obtained repeatedly on relatively small series of mice, and much less time and smaller animal colonies are needed for such experiments.

From the practical point of view of the chemotherapist, the important factor is the correlation between the response of human leukæmias and any other experimental system. It is not really important whether or not the experimental leukæmia resembles human leukæmia morphologically. In fact, if a bacterial system could be discovered which would show a very close therapeutic correlation with human leukæmia, because of its simplicity it would be much more valuable than any mouse leukæmia screening programme.

also noted by Law in 18 cases of spontaneous leukæmia in the C58 and RIL inbred strains of mice (15).

There are several useful agents that can be considered in the group of alkylating agents. The first of these to be used was the nitrogen mustard, methyl-bis (β-chloroethyl) amine, known by the code name HN2. Its use stemmed from wartime research of the Chemical Warfare Service on the toxic effects of these vesicant agents on the bone marrow and lymphoid tissues (16, 17, 18, 19). In patients with chronic myelocytic leukæmia, remissions lasting from one to six months may be achieved by a course of HN2 therapy and this may be repeated several times. Remissions may also be achieved in chronic lymphocytic leukæmia, but no beneficial effects can be expected in patients with acute stem cell leukæmia (20).

The nitrogen mustards have also shown definite effects in chloroleukæmia 1394 in the AK mice and to a lesser extent in the lymphoid leukæmia 9421 (13) The lymphosarcoma $6C_3HED$ was inhibited by the injection of nitrogen mustard in either intact or adrenalectomized animals by the administration of the mustards—a fact which tends to show that this effect is not mediated through the adrenals (21).

Triethylene melamine (TEM) causes remissions in the chronic leukæmias in much the same way as the nitrogen mustards, but has the advantage that it causes little or no nausea and vomiting and may be given by mouth as maintainance therapy (22). TEM appears to act in mouse leukæmia in a fashion similar to the nitrogen mustard HN2 and causes an increase in survival time in AK4 leukæmia in the AKR strain (23) and inhibits growth of the 8-azaguanine resistant variant of L1210 in the DBA strain (24)

In a report originally submitted in 1942 and published in 1944, Heilman and Kendall showed that cortisone had a markedly inhibitory effect on the growth of a transplanted lymphosarcoma and would even cause temporary regressions of tumours which had grown to 30 per cent of the total weight of the mouse (25) When the tumours returned, however, they were completely refractory to further cortisone

which were irradiated by the use of a radium source. Similarly, Kirschbaum *et al.* (5) have shown that in the F strain of mice at least a 50 per cent increase in survival time could be obtained in Line 15 myeloid leukæmia by irradiation.

Of the chemotherapeutic agents available for the treatment of chronic myelocytic leukæmia, arsenic was the first to be employed by Lissauer in 1865 (6). When given orally in the form of Fowler's solution, this compound will produce definite remissions in the early stages of the disease (7). Its administration, however, is often accompanied by nausea and vomiting and for this reason cannot be tolerated by some patients. Many strains of leukæmia can be inhibited by potassium arsenite. Among these are leukæmia 106 in the AK mice (8), and myeloid Lines 15, 686, RE, and 86 in F strain mice (5). Line I in the C58 mice is also inhibited by this agent (9).

Clinically, benzol has been employed extensively by Kalapos (10) and Koranyi (11) who reported excellent results in the treatment of the early stages of chronic myelocytic leukæmia. This compound was shown to be a highly effective agent in increasing the survival time of AK mice with chloroleukæmia 1394 and myeloid leukæmia 106 (8), and also caused some increase in survival in Line 15 of myeloid leukæmia in the F strain (5), and in Line I in C58 mice (9). It had no significant effects in myeloid Line 686, RE, or 86, or lymphoid Line 291 in F mice (5), or lymphoid Lines 1032 or 1630 in AK mice (8).

The ability of urethane to cause remissions in patients with chronic leukæmia was first demonstrated by Paterson *et al.* in 1946 (12). The oral administration of two to four grams daily of urethane induced remissions in a high percentage of these cases. It has also been reported to have a beneficial effect on patients with multiple myeloma and plasma cell leukæmia. Urethane has been shown to cause a prolongation of survival time in certain forms of mouse leukæmia, such as myeloid Lines 15, 686, and RE in the F strain (5), chloroleukæmia 1394 in AK mice (13, 14), and in Line I (9) and Line 825 (15) in the C58 mice. Definite palliative effects were

the hormones does not develop and the patients may be maintained for long periods of time on hormone therapy alone. In the chronic myelocytic leukæmia, however, there seems to be no beneficial effect from cortisone and there are many investigators who feel that in the granulocytic leukæmias of either the acute or chronic variety and in the monocytic leukæmias, ACTH and cortisone are actually contra-indicated.

The folic acid antagonists, Aminopterin and Amethopterin, were first successfully used in the treatment of leukæmia by Farber *et al.* (36) in 1948 in the treatment of children with acute leukæmia. Folic acid and citrovorum factor, its biologically more active counterpart, are vitamins necessary for the growth and maturation of normal erythroid and myeloid tissue of the marrow. Fortunately, from the point of view of the chemotherapist, they are even more necessary for the growth of the cells of some forms of acute leukæmia. The antagonists of folic acid, such as Aminopterin and Amethopterin, differ from folic acid mainly by the substitution of an amino group for a hydroxyl group in the 4 position of the pteridine ring Treatment with either of these agents causes a relative deficiency of citrovorum factor throughout the body which specifically damages certain types of leukæmic cells. By treatment with a folic acid antagonist, good hæmatological and clinical remissions, in which the child returns temporarily to normal health and in which the marrow has a total of 30 per cent or less of lymphocytes and blast cells, may be obtained in 30 per cent to 50 per cent of children with acute leukæmia. In a compilation of reports of the treatment of 425 children with acute leukæmia reported at the Second Conference on the Folic Acid Antagonists (37), 68 per cent were considered as having been improved by therapy (38). In our own series (39), 44 of 119 children, and one of 36 adults with acute leukæmia, experienced good hæmatological and clinical remissions after treatment with Amethopterin or other related folic acid antagonists. It is important to note at this time that only 37 per cent of the children responded to this

therapy. Other investigators have since demonstrated the inhibitory effects of adrenal cortical extracts (26, 27), cortisone (28), and Compound F (28) on transplanted and spontaneous leukæmias in the C58 and RIL (AKR) strains of mice, transplanted leukæmia of rats, and transplanted lymphosarcoma of mice. In other lines of leukæmia, large doses of cortisone have been shown to decrease the leukæmic infiltration and to lower the white count, but because toxic doses of the compound were necessary to hold the leukæmia in check, there was no increase in the survival time of these mice. In other strains absolutely no beneficial effect was noted, even at toxic doses of the drug (29).

The treatment of leukæmia with the hormones, ACTH and cortisone, was first reported by Pearson *et al.* (30, 31), and Farber *et al.* (32). A summary of the investigations conducted throughout the country was given at the Second Clinical ACTH Conference (33). In 80 of 175 children, and 14 of 60 adults with acute leukæmia, good clinical and hæmatological remissions were achieved. These remissions were characterized by a fall in the total leucocyte count to normal levels and a return of the marrow in most cases to a relatively normal functioning state. In our experience, such remissions were of relatively short duration, lasting one to twelve weeks, could not be repeated in adults, and could be repeated only a second time in children. Some investigators, however, have reported occasional third and fourth responses. As with all other agents used in the treatment of acute leukæmia, resistance develops also to the hormones; and resistance to cortisone is usually associated with resistance to ACTH and *vice versa.* On the other hand, there does not appear to be cross resistance between the hormones and the folic acid antagonists or mercaptopurine (34).

The hormones are also useful in the treatment of chronic lymphocytic leukæmia (30), particularly in those cases where there is an associated thrombocytopenia or hæmolytic anæmia. Pearson *et al* (35) feel that in chronic lymphocytic leukæmia, in contrast to the acute leukæmia, resistance to

the hormones does not develop and the patients may be maintained for long periods of time on hormone therapy alone. In the chronic myelocytic leukæmia, however, there seems to be no beneficial effect from cortisone and there are many investigators who feel that in the granulocytic leukæmias of either the acute or chronic variety and in the monocytic leukæmias, ACTH and cortisone are actually contra-indicated.

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drug and that many with what appeared to be morphologically identical disease did not respond, so that in children with this disease morphology may give no indication of response to the folic acid antagonists.

The folic acid antagonists have been studied in many strains of transplanted leukæmia in mice. Responses have varied from no effect in chloroleukæmia AK 1394 (40) and myeloid Line X in the F strain (41), lymphomas 1 and 2 in strain A mice (42), and L1858 in DBF_1 mice (42); to slight effect in myeloid Lines 15, 686, and 765 in F mice (41); to a doubling or tripling of the survival time, as seen in AK4 leukæmia (43); marked inhibition of growth of subcutaneous tumour masses and increase of survival time in L1210 in DBA mice (42); to frequent complete cures in Line I in the C58 mice (9).

Another antimetabolite, 6-mercaptopurine, has recently been shown to be useful in the treatment of acute leukæmia in both mice and patients. The synthesis of mercaptopurine was a result of a ten-year study of possible antagonists of precursors of nucleic acid by Hitchings, Elion, *et al.* (44, 45, 46, 47). In the studies on *Lactobacillus casei*, 6-mercaptopurine was demonstrated to be a purine antagonist although studies in animals have not shown that either the toxic manifestations or the anti-leukæmic effect of this compound can be reversed by simple purines The anti-tumour effect was first demonstrated by Clarke *et al* (48) in Sarcoma 180 in mice. These investigators reported that mercaptopurine not only inhibited the growth of Sarcoma 180, but actually caused complete regression of up to 30 per cent of such tumours. With this compound again there is a marked range in susceptibility among the various strains of leukæmia. The AK4 leukæmia which is so susceptible to treatment with Amethopterin is not affected in regard to survival time, but with very high doses of mercaptopurine a definite leukotoxic effect is noted on the leukæmic cells, with reduction in count. Line I, which is also very sensitive to Amethopterin, is relatively unaffected by mercaptopurine alone, but I/A (a variant of Line I which

has been made resistant to Amethopterin by many passages through treated animals) is quite sensitive to mercaptopurine and considerable increase in survival time may be achieved (9) Both Amethopterin sensitive and resistant variants of L1210 in the DBA mice are susceptible to mercaptopurine (9, 49).

This drug will produce clinical and hæmatological remissions in acute leukæmia in children and occasionally in adults (50, 51) in both previously untreated patients and those whose disease is resistant to Amethopterin and cortisone, but at least three weeks of therapy and often up to eight weeks are needed before these remissions are achieved. In 68 children with acute leukæmia, hæmatological and clinical remissions were achieved in 29 children (16 new, 13 previously resistant to cortisone or Amethopterin). Partial remissions with considerable clinical benefit and improvement in the peripheral blood occurred in 14 (five new, nine previously resistant to Amethopterin or cortisone), and 25 were considered failures (nine new, and 16 previously resistant to Amethopterin or cortisone) In 40 adults, five hæmatological and clinical remissions, six partial remissions, and 29 failures were reported. Remissions in such patients last from one to more than nine months, and patients who become resistant to mercaptopurine may still respond to the folic acid antagonists or to ACTH and cortisone. This compound is useful, not only in treating the acute leukæmias of children and adults, (including the monocytic variety), but also chronic myelocytic leukæmia. It also appears to have a unique therapeutic quality in that it has some very temporary beneficial effect even in the terminal acute stage of chronic myelocytic leukæmia. This particular stage has been unaffected by all other compounds previously used in the chemotherapy of leukæmia. In our experience, it is without beneficial effect in chronic lymphocytic leukæmia.

In addition to the fact that various strains of transmitted mouse leukæmia will respond to each of the nine different agents therapeutically active against human leukæmia, there is a close similarity *between the acute leukæmias of man and*

mouse in their ability to develop resistance to chemotherapeutic agents. Heilman and Kendall in their original report (25) noted that the lymphosarcoma which was initially so sensitive to the inhibitory effects of cortisone eventually became wholly resistant to this drug. The development of similar resistance in patients is all too familiar to clinical hæmatologists. Swartz and Robbins (52) demonstrated the ability of chloroleukæmia AK 1394 to become resistant to the effects of benzene. It has long been known in patients with chronic myelocytic leukæmia that although benzene is beneficial in the early stages, eventually the disease becomes totally refractory to such therapy.

The development of resistance to the folic acid antagonists has been studied in great detail (53, 54, 55, 56). In both mouse and human leukæmia, resistance eventually develops to these agents and there is complete cross resistance between all the 4-amino antagonists of folic acid so far studied. There is no cross resistance, however, to 6-mercaptopurine or cortisone in either species (9, 51, 57). Resistance to 6-mercaptopurine develops in both mouse and human leukæmias and here there is no cross resistance to the folic acid antagonists (49, 58).

In closing I would like to emphasize that strains of transplanted mouse leukæmia exist which will respond to every agent effective against clinical leukæmia. No one mouse leukæmia will respond to all the various agents any more than will any one type of human leukæmia. In the acute leukæmias in particular all the variations in responsiveness to the folic acid antagonists, the purine antagonists, and the steroids which are noted in the clinic are also seen in the mouse. There is often similarly in both species a lack of correlation between the morphology of the leukæmic cell and its biochemical properties as reflected in its response to chemotherapeutic agents.

Two agents of considerable value in the treatment of clinical leukæmia have been discovered through the use of mouse leukæmias and lymphosarcomas. These are cortisone and

triethylene melamine. The anti-tumour effect of 6-mercaptopurine was first noted against Sarcoma 180, but the effectiveness of this compound was subsequently demonstrated on mouse leukæmia before clinical evaluation was begun.

The value of a screening technique depends on its correlation and its simplicity and economy of time and effort. If bacterial or protozoal systems could be found whose response to chemotherapeutic agents closely paralleled that of human leukæmia, they would be preferable to mouse leukæmia because of the simplicity and economy of the technique involved. No such correlation has been discovered as yet, however, and therefore because of the close correlation which does exist between mouse and human leukæmia in regard to response to therapeutic agents, I submit that transplanted mouse leukæmia is today the best experimental tool for studying the complex problems of the chemotherapy of leukæmia.

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[Discussion of this paper was postponed until after the paper by Dr. Israëls.—Ed.]

THE SHORTCOMINGS OF ANIMAL RESEARCH IN LEUKÆMIA

M. C. G. ISRAËLS

ANY clinician who works in a department mainly interested in blood diseases has become conscious of the increasing frequency in his clinics of patients suffering from leukæmia. Part of this increase is undoubtedly due to better diagnostic methods; many cases formerly dismissed as "refractory anæmia" or "aplastic or aregenerative anæmia" will nowadays, after fuller investigation, be classified as leukæmia. Making a correct diagnosis is not necessarily a matter of academic tidiness, it has an influence on the methods we use in attempting to control the disease.

I think it is fair criticism to say that although it is over twenty-five years since the transmissibility of mouse leukæmia was established, the clinician, battling with the tragedy of human leukæmia, has derived little benefit from the immense amount of work that has been done, and the money expended, on the study of mouse and fowl leukæmia. Such new methods for the treatment of leukæmia as have emerged are not due to animal researches. Experimental mouse leukæmia is now used for "screening" new mitotic poisons, but the original stimulus for the use of such materials came from direct studies on human subjects, even the results of screening have to be treated with great reserve; it cannot be deduced that because a compound appears safe and effective in the control of mouse leukæmia that it will be equally safe and effective, or even effective at all, in human leukæmia of an apparently corresponding type.

The use of nitrogen mustards and the analogous compounds since developed sprang from the observation that the preparation of this substance as a war gas caused leucopenia in

exposed workers. The use of folic acid antagonists comes from observations on the leucocyte-stimulating properties of folic acid in states of malnutrition—and it is worth recalling that studies on rodents had given no hint that folic acid would have such a startling effect on human megaloblastic anæmia. (The work done in Prof. Witts' department on blind-loop anæmia in rats came later.) The use of cortisone in human acute leukæmia owes nothing to research in mouse leukæmia; in fact, I am not aware that results analogous to those seen in human leukæmia have been obtained in experimental mouse leukæmia.

The important facts that have been learnt from the voluminous studies on mouse leukæmia are briefly these:—

1. The genetic make-up of the host influences decisively the susceptibility to a transmitted leukæmia. This inherited susceptibility is often extraordinarily limited and may only be effective when one particular strain of leukæmia is transmitted.

There is, however, little evidence that heredity is of any practical importance in human leukæmia. Some leukæmic families are known, and it is odd that most of them have been found in Denmark by one worker (Videbaek, 1947). Although most clinicians know about this possibility and are on the look-out for other cases in the family, physicians outside Denmark have little to report. We have seen some 600 cases of leukæmia in Manchester in the past twenty years and, if Videbaek's incidence of familial involvement had occurred, we should have seen some 48 patients with at least one other member of the family affected. In fact, we have seen none

2 Exposure to doses of X-rays or other ionizing radiations sufficient to put the blood-forming tissues out of action for a time greatly increases the susceptibility to transmitted leukæmia in the mouse. Here there is some evidence that exposure to ionizing radiation can increase the incidence of human leukæmia. radiologists seem to be more liable to leukæmia than other doctors; an increased incidence of leukæmia has been reported in the surviving populations

exposed to atomic explosions in Japan. But here again, this factor can hardly be of practical importance in the majority of cases of human leukæmia.

3. The third fact concerns the changed properties of the leukæmia leucocyte. Furth (1951) and many others support the hypothesis that the essential change in leukæmia resides in the leukæmia cell and consists of an acquired inability of immature leucocytes to respond to forces normally regulating their proliferation and maturation. "The change," Furth says, "is essentially that termed neoplastic; the end-result is a new type of cell with a wide range of fixed abnormalities as concerns behaviour and appearance."

Now every clinician is familiar with cases of leukæmia that seem to exhibit this fixed property of immature leucocytes; the case is usually one of acute leukæmia, there are numerous myeloblasts—or other immature cells—in the peripheral blood and bone marrow; the course is little influenced by treatment, and at post-mortem much infiltration of the liver, spleen, lymph glands and other organs is found. In transmitted mouse leukæmia, Engelbreth-Holm (1942) has pointed out in what a large proportion the disease is in an aleukæmic phase for much of its course; in human acute leukæmia too, this feature is common. In transmitted mouse leukæmia the cells remain fixed in type, but tissue culture methods applied to human acute leukæmias show that these cells are *not* fixed in type. My original idea was to obtain tissue cultures of human leukæmic leucocytes from bone marrow so that their properties could be thoroughly studied. Although I was careful to choose patients whose marrow contained predominantly primitive cells—say myeloblasts with no detectable myelocytes on the smears—I always ended up with a culture containing myelocytes and not myeloblasts, so I could no longer be sure that the cells differed from normal (Israëls, 1940). These results were reproduced some years later by Hoogstraten working in Sir Lionel Whitby's department. Engelbreth-Holm has suggested as an explanation that some cases of human leukæmia may not be "fully neoplastic"—

but such an explanation does admit that mouse and human leukæmias may not be so analogous as they seem.

In recent times the piece of new knowledge that is most striking to the clinician is the fact that it has proved possible to induce a remission in some patients with leukæmia by giving cortisone. Spontaneous remission in leukæmia has been known for many years, especially in children, and such remissions may last for some months—the longest I have seen is five months. During the remission it may not be possible to detect, from blood or bone marrow, that the patient has ever had leukæmia. Instances of spontaneous regression are known in both animal and human tumours and the existence of spontaneous remission in leukæmia cannot be quoted as evidence against the neoplastic theory of leukæmia. But the cortisone-induced remissions in human leukæmia are not, in my opinion, coincidental spontaneous remissions; they occur too frequently and the pattern is different. Most of the prolonged and complete cortisone remissions that we have seen occur in the subleukæmic lymphatic leukæmia of children. This condition often presents clinically more like an aplastic anæmia than a leukæmia: the child is anæmic, there may or may not be moderately enlarged lymph glands and spleen; the leucocyte count is low and there is a variable preponderance of mature lymphocytes; marrow puncture shows a cellular marrow, most of the cells being lymphocytes in various stages of maturity, but there are only few lymphoblasts. The onset of a cortisone-induced remission is usually signalized by a rise in the platelet count, followed by a *rise* in the leucocyte count and the appearance of a more and more normal differential white cell count There may be a concomitant rise of reticulocytes, and lastly the hæmoglobin and the red cells rise to normal levels. Bone-marrow examined at this stage will show quite a normal active picture. Once the remission is induced, it does not seem to matter whether cortisone is given or not; it seems to have little influence on the occurrence of relapse, and in our experience, as in that of others, relapses are much more likely to be resistant to

treatment. Out of 45 cases of acute leukæmia treated with cortisone, we have seen 17 partial or complete remissions lasting from a few weeks to many months. In other forms of acute leukæmia cortisone induces only few remissions and they are rarely complete or prolonged.

Now, so far as I am aware, this effect of cortisone has not been reproduced in the transmissible lymphatic leukæmia of animals. The effect of cortisone in animal leukæmia, according to published work (Kirschbaum, 1951), is to retard the rate of spread of the disease, but no more. The effect is much more like that seen in the human condition that I have called elsewhere lymphoid reticulosis. In this disease the emphasis is on organ involvement rather than on the blood changes; the patient has enlarged lymph glands, especially in the abdomen and the mediastinum, and enlargement of the liver and spleen, but the peripheral blood shows no more than a mild anæmia and a polymorph leucocytosis. The histology of the glands shows infiltration with small lymphocytes with loss of normal architecture. Quite soon excessive lymphocytes occur in the bone marrow, but a leukæmic picture in the peripheral blood comes at the end of the course of the disease. Cortisone given to these patients merely reduces temporarily the size of the gland masses without influencing the course of the disease. In fact, I think that transmissible lymphoid leukæmia of mice is much more analogous to this human lymphoid reticulosis than to the true leukæmias; the human reticulosis can end sometimes as a true lymphosarcoma, as the mouse leukæmia seems to do.

The fact that cortisone produces a fundamentally different effect in human and transmitted mouse leukæmia confirms the clinicians' suspicion that the animal researchers are not studying a really analogous disease. The important thing to do now seems to be to find out how cortisone induces these remissions—and transmissible mouse leukæmia appears unsuitable for this study.

If clinicians have been relatively quiet about their misgivings about the value of contemporary animal research in

leukæmia, it is for several reasons. One is that the neoplastic theory answers many of the questions about leukæmia and still forms the theoretical basis for much of the treatment applied; another is that it has proved very difficult to get evidence that human leukæmias *are* significantly different from the transmissible mouse leukæmias; a third reason is that clinicians have been unable, till now, to point to any alternative line of research that promised useful results. The cortisone effect at least suggests an alternative.

The study of mouse leukæmia, and fowl leukæmia, seemed to have reached an impasse, and the literature suggests that these conditions have been studied in late years more for the light they might throw on the general properties of tumours than for any hope of elucidating the nature of human leukæmia.

It is, therefore, very encouraging to find that these doubts have at least spread to some workers in the animal field. These doubts have come about because of the recognition of differences between "dependent" (or conditioned) and "autonomous" neoplasms—the subject on which Dr. Furth is to speak this afternoon. Furth (1953) has recently suggested that there exist grades of leukæmia: those with seemingly normal cells and those with fully autonomous cells. In animals the only type that has been studied so far is the type in which the leukæmic cells are fully autonomous, and are capable of overwhelming the regulatory mechanisms in the host and proliferating until death of the host Analogous types of leukæmia do occur in man, as I have pointed out, but they are not common, nor are they the types that we have been able to influence by treatment. Furth states that in some human leukæmias the cells are "immature but not altered and may lack autonomy." And he admits that though such leukæmias do occur in animals they have not been adequately studied

So the clinician is justified at last and he is encouraged by another aspect of this problem that fits in with his experience. This is the work of Huggins and his co-workers (Huggins

and Bergenstal, 1952) which shows that control of the hormonal environment can prevent progression of dependent tumours to autonomy. Is this perhaps the way in which cortisone works in leukæmia?

For the first time for many years the clinician sees some ray of hope that help may come from the study of animal leukæmia. But it is clear that these studies will be so different from those made so far, that time must elapse before we can hope to see results.

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DISCUSSION

WITTS: This discussion I think should primarily be on the question of the analogy of experimental and human leukæmias. Prof. Engelbreth-Holm did raise a number of other issues, but they could perhaps be postponed.

WHITBY: As the result of work in my department, I have concluded that the case for the malignant nature of leukæmia, and therefore for

6-mercaptopurine, which arose originally from animal experiments; my experience has been much the same as his, that 6-mercaptopurine is the only treatment that I have found to have any effect in acute monocytic leukæmia in the human subject.

ENGELBRETH-HOLM: Dr. Israels mentioned that I had said that some

of man which are analogous to the mouse leukæmias are the autonomous,

the frank tumorous cases. But it is obvious that some of the clinical cases called leukæmia today are certainly not analogous to what we see in mice. Dr. Israëls in that case used the term leukæmia in the wrong way.

ISRAËLS: It depends what you mean, doesn't it? From a purely clinical point of view, we wouldn't mind so much what happened to the relatively few cases with fully autonomous leukæmias if only we could deal with the much larger numbers which don't appear to be autonomous. The idea of the Lady Tata Memorial Trust was to find some means of [illegible]

[illegible]

leukæmia. Twenty years ago Castle studied the gastric juice in leukæmia because of some such resemblance to a nutritional deficiency.

HADDOW: We are indebted to Dr. Israëls for his provocative paper and point of view, but it would be a pity to regard these matters as at all exclusive Surely the study of the whole subject would be set back if we were deprived of the animal leukæmias, and equally if we were to obtain no stimulation or information from the human side.

The case of urethane has always interested me in this connection. The work was inaugurated entirely from the aspect of its action on animal and plant cells, and only later did the substance's interesting properties seem to justify clinical trials. These proceeded for nearly three years before its application to human leukæmia was detected by Dr Paterson

Dr. Israels expressed disappointment that after the expenditure of so [illegible]

quietly as he himself would wish, but as a matter of policy we must decide to what extent this is a crusade, designed to lead to some practical contribution. I think we must welcome information from any field, human and animal And as far as the underlying chemical differences

between normal and leukæmic cells are concerned I fail to see how we can ever elucidate these from human material alone.

ENGELBRETH-HOLM: Another thing that Dr. Israels mentioned was the work of Dr. Videbaek on the heredity of human leukæmia We are not convinced that Videbaek is right. He collected two hundred cases of leukæmia and two hundred without leukæmia, very similar groups, and then investigated their families. If he had only investigated the parents and siblings, it would have been all right, but he took in more distant relatives. If you cut them out, there is no proof of heredity in the paper.

LAW: Considering the heterogeneity of the human population, it is not

JACOBSON: I have a question which in my own mind is basic to the

we attack it.

FURTH: I should like to underline what Dr. Jacobson says, namely our profound ignorance with respect to the relation of the reticulum to the more mature hæmopoietic cells. In the neoplastic experimental leukæmias of animals, the alteration concerns the myeloblast and the lymphoblast, which multiply without maturation or with limited maturation, and the altered cell perpetuates itself in the course of continued subpassages. But this is not the whole story Decrease of leukæmia incidence by treatment with cortisone cannot be caused by virtue of destruction of adult lymphocytes, because they live but a few hours, and it seems that a change in the thymic reticulum is the key to the problem of leukæmia induction by irradiation and leukæmia prevention by cortisone.

FAGRAEUS: Have the workers who found an effect of cortisone on mouse leukæmia any proof as to whether it is an effect on the differentiated cell or a prevention of the differentiation of the reticulo-endothelial cells? So far we have found that cortisone has no effect on antibody-forming cells once they have reached a certain stage of differentiation; it has to be given before the antibody production has really started.

and mouse leukæmia, mentioned that in mice you get a transplanted leukæmia by graft, but only in animals with the same genetic constitution, and in humans you would have to transplant it to identical twins. Now in mice you can transplant it from one strain to another, provided that you use newborn animals. To my knowledge no attempt

has been made to transmit human leukæmia to a newborn baby—nobody would think of doing it—but attempts to transplant leukæmia to adult individuals have failed. Similarly in mice, attempts to transplant leukæmic cells or blood, to adult mice of a different strain, in most instances fail also.

BURCHENAL: It seems to me that we have two possible ways of

[illegible]

try to evaluate a known compound in the clinic; it requires many

[illegible]

BURCHENAL: That is particularly true of the nutrition of some of

[illegible]

precursors

[illegible]

treatment of acute leukæmia in children with folic acid antagonists. I believe this was based on Farber's experimental work on transplanted sarcomas in folic acid deficient rats, and also on work of Lewisohn and collaborators

[illegible]

tion of the disease. For that reason he requested the folic acid antagonists rather than folic acid conjugates There is no doubt that the Lewisohn experiments led to the folic antagonists, but in a rather roundabout way.

DE BRUYN. Because it is so difficult to do experiments with patients, it might be helpful to cultivate the tissues of patients. The effect of drugs and irradiation could then be studied on human tissues as well as on mouse tissues cultivated *in vitro*.

KIELER: In spite of the efforts of several investigators we have no satisfactory method for the cultivation of human bone marrow *in vitro* Only survival for a limited period has been obtained so far. However,

[illegible]

dividing for two weeks, but how long it will go on I don't know.

ENGELBRETH-HOLM. Do they differentiate?

KIELER· Yes, but they may stop later on—I don't know.

BURCHENAL· There is evidence of growth?

KIELER: There is evidence of growth, mitosis, and increase in the number of cells.

WITTS: Dr Bessis, you did some very interesting work on the study of remissions in the human being after large transfusions

BESSIS: We have abandoned the large exchange-transfusions, but the results are quite clear After large blood exchange-transfusion, one sees 30 per cent remissions. We assumed that there was a maturation factor in the plasma, but this was definitely not true, because before the

[illegible]

DUSTIN. Dr. Burchenal mentioned benzene as one of the agents capable of being used in the treatment of leukæmia, but I think all

[illegible]

carbons, a carcinogen or not?

OBERLING. We found that benzene had no effect on rats even with very large doses.

ISRAËLS· I work in an industrial area where there are large chemical works, and benzene is much used in the rubber industry. If anyone in the rubber industry ever gets leukæmia, immediately this paper is referred to and one hopes to get compensation. We have never been able to establish it, usually the man has stopped working with benzene ten, fifteen or twenty years before. It doesn't seem as if benzene workers get leukæmia any more than people who work in other parts of the factory. The Industrial Health Unit at Manchester has tried to go into this matter.

FURTH: Are benzene-induced leukæmias myeloid?

ISRAËLS. They are said to be lymphatic.

FURTH In spite of a more specific action of benzene on the bone marrow?

ISRAËLS· That is said. But the ones I have seen have oddly enough been lymphatic.

[illegible]

controls had no lymphosarcoma at all. So I think there is some factor in benzene which stimulates neoplasia. On the other hand, in Switzerland, restrictions for benzene workers are very well controlled, we use it very little Out of about 450 leukæmias we have seen in the medical department, 3 cases were in benzene workers one acute myeloblastic leukæmia, one chronic lymphatic, and one chronic myelogenous I think there is no doubt, if you go through the literature, that there is about nine to ten times more leukæmia in benzene workers than in the normal population

ENGELBRETH-HOLM It might be a good idea to try to accelerate the development of leukæmias in a strain with about 10 per cent spontaneous leukæmias It is a much more sensitive way of testing than to produce them in a strain with no leukæmias. Prolonged treatment in mice of that kind would perhaps answer the question

FURTH In C57 Black mice, the incidence of leukæmia is about 1 per cent, and this can be raised by ionizing irradiations to 5–70 per cent, depending on the method of administration. Most induced mouse leukæmias originate in the thymus There are, however, strains of mice in which ionizing irradiations produce myeloid leukæmia, so I would not be surprised if, with proper selection of strain and technique, the leukæmogenic power of benzene could be demonstrated

THE CONCEPT OF CONDITIONED AND AUTONOMOUS NEOPLASMS

J. FURTH

Ability of unrestrained proliferation was at one time considered a distinguishing feature of neoplastic cells until it became known that many if not all normal cells are capable of unrestrained proliferation in tissue cultures. Obviously there are restraining forces in a complex organism containing hundreds of different types of cells keeping each within a wanted limit, as there are specific stimulating forces which, in case of deficiency, raise the number of these cells to the desired level. If this physiological homeostatic ("feedback") mechanism is deranged, and the balance is steadily tilted to dominance of the proliferative forces, a tumour-like growth may result. Recent experimental studies have disclosed the existence of several such situations, of which I shall mention a few:—

(*a*) *Pituitary tumours secreting thyroid stimulating hormones* can be produced by destroying the thyroid and thereby creating a sustained need for thyroid hormone (Gorbman, 1949). These tumours can be grafted on hosts whose thyroid is depressed, and in such hosts they grow, metastasize, and are as fatal as common cancers, but they cannot be grafted on normal hosts (Furth and Burnett, 1951). Such conditioned tumours can be controlled by administration of the physiological restraining force, the thyroid hormone. Conditioned tumours, if sustained, frequently give rise to autonomous cancers which grow in normal hosts and cannot be restrained by thyroid hormone, and are sometimes even stimulated by it (Gadsden and Furth, 1953).

(b) *Similarly thyroid tumours can be induced by blocking* TH synthesis by anti-metabolites, which do not abolish

responsiveness of the epithelial cells of the thyroid to the thyrotrophic hormones of the pituitary which stimulate them, and this sustained stimulation leads to the development of thyroid tumours. Such tumours can invade lymphatics and metastasize to the lung, but can be grafted only on hosts whose thyroid-hormonal synthesis is similarly blocked. Thus they are conditioned neoplasms, but, when sustained, they can give rise to autonomous cancers (Bielschowsky *et al.*, 1949, Morris *et al.*, 1951).

(c) Administration of stilbœstrol causes the development of pituitary tumours which grow in hosts similarly conditioned by this œstrogen but not in normal hosts (Dunning *et al.*, 1947).

Using a different technique, Greene (1951) has demonstrated that in the course of their development tumours may acquire autonomy not present at their inception.

While the growth stimulating and restraining forces are best known in the domain of the endocrines and their target organs, it can be presumed that the underlying principle holds for most other cell types. It is certain that the leucocyte levels are well regulated in normal hosts, and it can be presumed that there are specific forces which stimulate or restrain their production and regulate their delivery. On the basis of events with other regulated cells it can be postulated that a permanent disturbance of the homeostatic balance might result in leukæmias in which the proliferating cells are essentially unaltered, and which could be controlled at their inception by restoration of the deranged equilibrium of the regulatory forces

Earlier puzzling observations on some leukæmias can be explained by the supposition that they were conditioned growth disturbances. I shall cite a few.—

(a) Primitive cells of acute leukæmias of man have been stated to mature rapidly in tissue cultures. This points to the presence of a maturation factor in the normal plasma, or to the existence of blocking or restraining factors in leukæmic hosts. These experiments deserve renewed study. If

confirmed, they may yield an assay technique of a physiological force regulating hæmopoiesis.

(b) Several investigators studying the metabolism of human leukæmic cells concluded that premature hæmopoietic cells from leukæmic and normal hosts behave alike.

(c) Many hæmatologists have pointed to difficulties—even to the impossibility of distinguishing immature hæmopoietic cells in some leukæmias from immature, normal, hæmopoietic cells. The common cytological criteria of malignancy do not apply to immature cells of many leukæmias.

(d) The difficulties of distinguishing between extensive extramedullary hæmopoiesis and chronic myeloid leukæmia in mice are well known. Years ago two associates (Barnes and Sisman) described their distinctive features, and subsequently we have attempted to obtain a biological differentiation between myeloid leukæmias and non-malignant extramedullary hæmopoiesis by means of transplantation studies in normal hosts. This proved to be a long-drawn-out work because of scarcity of available material, the long latency period of most grafted chronic leukæmias, and the natural occurrence of both conditions in the same recipient strain. It is regretted that during and after the war other problems made us lose track of this one. While spontaneous leukæmias arising in the high leukæmia strain AK proved transplantable to every young member of the strain, chronic myeloid leukæmias arising in the equally inbred RF strain did not. In retrospect, it seems that some of these chronic myeloid leukæmias may have been conditioned neoplasms Attention should be directed to those types of chronic leukæmias which are not transplantable in the highly inbred strain of origin. I propose the working hypothesis that if a neoplasm arising in an inbred strain is not transplantable in that strain it should be presumed to be conditioned

When it was discovered that leukæmic cells of inbred strains of mice are altered cells, and that a single one of them will proliferate in a new host, causing leukæmia, we searched enthusiastically for, and studied, graftable leukæmias of

different types, ignoring warning signs that transplantability in normal hosts may not be universally true for the leukæmias. I was among those who ignored the wiser colleagues who have argued that what we call a neoplasm or a tumour is a state in which cells proliferate either because of alterations intrinsic in the cells or alterations in the host.

Whether conditioned leukæmias actually exist, and, if so, how frequent they are, only future studies will tell. This hypothesis is, I believe, worthy of further special experimentation. A spontaneous neoplasm can be certified to be conditioned, but autonomy can only be guessed at. Autonomy is a relative and quantitative and ever uncertain characteristic.

Years ago, when I came to the conclusion that the mammalian leukæmias then studied were composed of permanently altered cells, I was depressed by the consequences of this conclusion, for control of leukæmia, like that of common cancer, would call for an agent which selectively destroys all leukæmic cells without harming their normal prototype. The hypothesis that some leukæmias are conditioned neoplasms has at least one virtue: it raises the hope that some leukæmias might be controlled by restoring the normal balance, and may lead to renewal of much-needed research on forces regulating normal hæmopoiesis.

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DISCUSSION

GORER. Dr Furth spoke about failure of transplantation in an inbred strain as the criterion for a conditioned neoplasm. I am not sure that is

a proper criterion. I believe I can transplant normal bone marrow, producing in mice something which anatomically I call bone marrow.

There is another point which I think is technically important in screening. It's not that you are screening on transmissible leukæmia,

ous leukæmia, the standard

tumours like the Walker carcinoma.

BURCHENAL: I think that is a very important point. Line I leukæmia,

ENGELBRETH-HOLM: Did I understand, Dr Furth, that no morphological differences were found between the normal marrow cells and the leukæmic ones?

FURTH: Some transplanted leukæmias are composed of immature cells with no known cytological features which would enable them to be distinguished from normal immature cells

ENGELBRETH-HOLM. Does that include the measurements of the nuclei?

FURTH: Yes.

ENGELBRETH-HOLM: Because in 1932 Heiberg in Copenhagen measured the nuclei from different human leukæmias and compared

Arley who tried to apply quantum theory to tumour development? They think that, instead of a gradual change from normal to malignant, there are three or four or more sudden stepwise changes in the life of the nucleus.

FURTH: I have no doubts, on the basis of transplantation studies, that there are stepwise changes. When you begin with a spontaneous case of leukæmia and transplant it from animal to animal, you will find that the latency period is likely to become gradually shorter,

forces which restrain that cell? The answer was yes, these cells do not grow in the presence of the normal restraining force. Nevertheless, [illegible]

chromatin in normal cells from that in leukæmic cells, both in mitotic and in interphasic nuclei.

OBERLING: Büngeler (*Verh. dtsch. path. Ges.*, 1952, 35th Session, p. 10) has made a very good survey of this subject, and has given many examples of tumours which are conditioned and autonomous.

FURTH: My review on the subject (*Cancer Res.*, 1953, 13, 477) cites many instances contained in Büngeler's earlier article (*Ztschr. f. Krebsforsch.*, 1951, 58, 72). Büngeler supposes that the majority of benign tumours are such conditioned growth disturbances. I agree with him, and experiments should be directed towards finding out, for example, to what extent disturbances of calcium and phosphorus metabolism can influence parathyroid growth, or electrolyte disturbances adrenal growth. Büngeler's observations are based almost exclusively on human anatomical findings—ours on experimental studies in animals—a reminder for those who worry about the use of animals in the study of human neoplasms.

[illegible]

X [illegible]
nc [illegible]
cc [illegible]

stage of development.

ISRAELS: I would have said that it is practically impossible to differentiate between things like lymphoblasts, myeloblasts, or monoblasts, and I think people who say they can are deceiving themselves. So far

as we know, if you get a sufficiently primitive cell they all look exactly alike.

ENGELBRETH-HOLM: That is true for the methods hitherto applied.

FURTH. There are other matters to be considered, such as the rate of multiplication. If the cell multiplies rapidly or is overstimulated, morphological abnormalities may appear. There are also nuclear differences between an embryonal cell and its adult homologue.

ISRAËLS: A lot of histochemical methods have been produced, but if you put a cell under the microscope and ask "Is this a lymphoblast, a myeloblast or a monoblast?" it's a pure matter of chance what answer you get. I've tried many histochemical methods for this purpose but none give better results than properly performed standard methods.

GORER: There is also the question of the way they move in tissue culture. Wintrobe, and I think Lewis and Rich were able to differentiate primitive cells in this way.

ISRAËLS: Human tissue culture is a very difficult procedure, as everyone who has tried it has found to his cost. You so often end up with cells you aren't interested in.

THE METHOD OF QUANTITATIVE DETERMINATION OF FOWL LEUKÆMIA VIRUS

ASTRID FAGRAEUS

WHEN starting the investigations on the distribution of the leukæmic agent in the cell we were faced with the problem of how to determine the virus quantitatively in various fractions. As no serological methods existed, it was obvious that we had to titrate the infectious capacity and judge the virus content by the infectivity titre. We proceeded in the usual way for determining virus infectivity in tissue material by using tenfold dilution steps in order to estimate the 50 per cent endpoint, according to the method of Reed and Muench.

When reviewing the literature we found that rather few quantitative assays in this field had been made, and the ones published did not seem very promising for the application of the Reed and Muench estimation technique, as they showed a very large spread of infectivity incidence over many dilutions, and usually gave no 100 per cent take in the lowest dilution. Furth (1932) published some series on the concentration of erythroblastic leukæmic agent in whole blood, washed blood cells and plasma The test animals were four- to five-month-old chickens, and the highest percentage of takes was 71 4 for whole blood, the titrations showed a spread over a very wide range, with sometimes a higher percentage of takes in a lower dilution. Later, in 1951-52, the Beard group published rather extensive studies on the dose-response relationship in avian erythromyeloblastic leukosis (Eckert *et al.*, 1951, 1952). They used three-day-old chicks and a very impressive number of animals in each group. The injection dose was 0 1 ml. As shown in their tables, the incidence of

positive inoculations was spread out over a very wide range, and a dilution of a thousand times only decreased the incidence from about 60 to 20 per cent. On the other hand, they found *a linear relationship between dose in log ml. of plasma and* the reciprocal of the latent period, and that relationship was useful for the bioassay of leukosis virus. They write that "no point of reference has yet been devised, such as the 50 per cent endpoint employed with many viruses, for comparing the levels of infectivity of different preparations containing the erythromyeloblastic leukosis virus" (Eckert *et al.*, 1952).

Our experience after many titration series using different material is not quite as negative. We have on the contrary found it possible to use the 50 per cent endpoint determination.

Material: We used an erythromyeloblastic strain kindly provided by J. Engelbreth-Holm.

Test animals: Five- to eight-day-old chicks from an inbred Leghorn strain.

Titration: Tenfold dilution steps of the various extracts and suspensions were made, using in the last experiments 10 per cent normal rabbit serum as a diluent. 0·5 ml. of each dilution was injected intravenously into five to six chicks.

Preparation of cell material: Spleen tissue from leukæmic chickens was cut in small pieces, passed through a metal screen and suspended in saline or Tyrode solution. The fragments in the suspension were eliminated by repeated filtration through sterile gauze. The loose cells were destroyed either in a glass homogenizer or in a Waring Blendor, as indicated in the Tables. These suspensions are referred to as total suspensions. In some experiments the spleen tissue was cut directly in a Waring Blendor. Centrifugation at 3,500 r.p.m. followed if tissue extracts were to be examined.

Results

Our results showed no certain evidence for a seasonal variation, often referred to in earlier publications. As can be further seen in the Tables, we often found a 100 per cent take

Table I

PER CENT TAKES AFTER INOCULATION OF TISSUE MATERIAL CONTAINING AVIAN ERYTHROMYELOBLASTIC VIRUS INTO FIVE- TO EIGHT-DAY-OLD CHICKS

Exper	*Month*	*Material injected*	*Waring Blendor or Homogenizer*	*Centrifuged*	*Per cent positive*	
					1st dil used	*2nd dil Dil factor = 10*
15	April	Total suspension	W.Bl	–	100	83
23	June	Total suspension Cytoplasm	H H	– +	80 80	100 40
28	October	Total suspension Cytoplasm	H H	– +	100 80	40 100
29	December	Total suspension Cytoplasm	W Bl H	– +	83 80	20 20
35	October	Total extract (10–2 7)	W Bl.	+	72	33
30	October	Total suspension	H	–	84	33
63	October	Total extract	W Bl	+	100	100
10 (Pontén)	October	Cytoplasm	W Bl	+	100	100

Table II

TITRATION OF AVIAN ERYTHROMYELOBLASTIC VIRUS IN VARIOUS MATERIALS. 5–6 FIVE- TO EIGHT-DAY-OLD CHICKS WERE INJECTED IN EACH GROUP.

Exper	*Material injected*	*Centrifuged*	*W.Bl. or H*	*Per cent positive Dose in 10 log dilution*						
				0	—1	—2	—3	—4	—5	—6
28	Total suspension	—	H	—	100	40	50	20	25	—
	Cytoplasm	+	H	—	80	100	20	60	—	—
36	Total suspension	—	H	—	84	33	33	17	0	
29	Total suspension	—	W.Bl.	—	—	83	20	0	0	—
	Cytoplasm	+	H	—	80	20	0	0	—	—
	Nuclei	—	H	60	33	40	50	0	—	—
16 (Pontén)	Cytoplasm	+	W.Bl.	100	100	80	20	—	—	
	Mitochondria	—		60	80	40	40	—	—	
63	Total extract	+	W.Bl.	—	100	100	100	60	17	0

in the lowest dilution used. In some of the experiments, however, we did not consider it necessary to use the lowest dilution possible, and thus did not get a 100 per cent mortality in the first group. It is interesting to see how often the infectivity incidence was higher in a lower dilution group. We found that this rarely occurred when we were dealing with virus material homogenized in a Waring Blendor. An uneven distribution was more frequent when we used cell suspensions treated with the less effective cell destroyer, i.e. the glass homogenizer. When a rather inhomogeneous virus material, such as the so-called nuclei fraction or mitochondria, was injected the irregular spread of incidence was quite typical.

Discussion of results

We have thus found a certain deviation from earlier titre figures published, in that we mostly have a 100 per cent incidence in the first dilution, *if* this is low enough. We also have evidence for the assumption that the distribution of incidence in the different dilutions varies with the material injected. This has been shown earlier for whole blood, but in this case it was assumed that the leukæmic cells proliferated in the new host We get this uneven distribution also with destroyed cell material, when this could be expected to be inhomogeneous. What explanation can we find for the difference between our results and those published by, for example, the Beard group?

1 The virus strains are different. There is *inter alia* a great difference in the incubation period. The latency time in our experiments is only eight to twenty days (Table III), whereas it varies from twenty to seventy-two days in the experiments of the Beard group

2. The susceptibility of the host might vary much more in their experiments. A certain resistance to virus has been postulated, though Rothe Meyer (1934) pointed out that resistance could never be found in chickens younger than eight weeks.

Table III

LATENT PERIOD AFTER INJECTION OF DILUTIONS OF VIRUS (SPLEEN TISSUE EXTRACT, EXPER. 63).

0·5 ML. OF EACH DILUTION INJECTED INTO EIGHT-DAY-OLD CHICKS

Days after injection	*Dilution*					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
9	1					
10	2	4				
11	1	1	2			
12	2	1	2	1		
13				2		
14			2			
15					1	
16						
Positive out of chicks injected .	6/6	6/6	6/6	3/5	1/6	0/6

3. The dose varies. We inject 0·5 ml. and the Beard group gives 0 1. It should be preferable to inject 0·5 ml., as that diminishes considerably the likelihood of a technical error.

4. The virus material used by the Beard group might be inhomogeneous. There is no certainty that the virus is evenly distributed within the plasma. They have a long incubation period and a rather long time elapses from the appearance of first leukæmic cells in the blood to the time when the animals die. In a later paper the Beard group discusses the fact that the plasma from chickens with a very long incubation time has a very low infectivity titre in spite of the fact that the plasma contains the particles they assume to be virus. They suggest the presence of some inhibiting material in the plasma.

Summary

1. The titration of avian erythromycloblastic virus in five- to eight-day-old chicks has mostly given even titration series permitting the infectivity titre to be estimated according to the method of Reed and Muench.

2. Some irregular titration series were found. The connection between these series and the material injected is discussed.

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[Discussion of this paper was postponed until after the paper by Dr. Thorell.—Ed.]

STUDIES ON THE ENDOCELLULAR DISTRIBUTION OF THE TRANSMISSIBLE FOWL LEUKÆMIA VIRUS

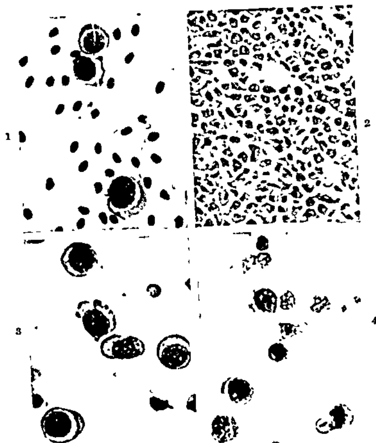
Bo THORELL

THE primary task when starting our studies on fowl leukæmia was the localization of the transmissible agent within the leukæmic cell. From earlier cytochemical analyses of blood and bone marrow cells we knew that leukæmic cells exhibit some characteristic properties, for example their content and distribution of nucleic acids (Thorell, 1947). From the point of view of cellular and virus biochemistry many questions present themselves. For instance, do these nucleic acids totally or in part belong to the virus (or the cell)? Do we have two different metabolic systems which can be studied separately? If so, we should be able to investigate the relationship between cell proliferation and virus synthesis.

When separating the different structural constituents of the leukæmic cell it is impossible to exclude contamination of one fraction by another. Thus it is clear that the determination of the virus activities in the different fractions must be quantitative. The paper of Dr. Fagraeus has given you some idea of the possibilities of quantitative leukæmia virus determination when using our strain under our experimental conditions.

As regards the preparation of the leukæmic cells, I will only mention a few points, which are being published in more detail elsewhere (Fagraeus and Thorell, 1954).

Fig. 1 shows the erythroleukotic cells in a smear from the peripheral blood. Fig. 2 is a section from the greatly enlarged spleen, the parenchyma of which is filled with leukæmic cells. Fig. 3 shows a smear from a splenic cell suspension in Tyrode salt solution. Such suspensions were



Figs 1–4. See Text.

treated in a plastic-glass homogenizer at high speed until many cells had lost their cytoplasm (Fig. 4). Counting the cells and nuclei before and after the treatment revealed no significant destruction of nuclei. After centrifuging at a moderate speed a clear supernatant was obtained, designated the "cytoplasmic fraction."

For comparison, a total cell suspension was homogenized in a Waring Blendor at high speed. In some experiments a preparation called "nuclei" was made as well by differential centrifugation. Both this and every cytoplasmic fraction were also treated in the Waring Blendor to ensure similarity of treatment. Of course all operations were carried out in the cold.

The virus activities in the different preparations were determined by titrations on eight-day-old chicks. Dr. Fagraeus has discussed the quantitative aspects of this (Fagraeus and Thorell, 1954) Representative results are given in Table I, which shows that the virus activity of the

Table I

EXPERIMENT 29

	Total cells	*Cytopl fraction*	*Nuclei*	*Sediment from homogenized susp*
Titre, 50 per cent end-point	$10^{-4 \cdot 4}$	$10^{-4 \cdot 4}$	$10^{-4 \cdot 2}$	$10^{-4 \cdot 4}$
Dry weight mg /ml	11 608	9 180	9 620	9 480
Kjeldahl N mg /ml	0 440	0 0335	0 116	0 180
Total P mg /ml	0 0460	0 00878	0 01271	0 01944
N/P	9 4	6 1	9 1	9 3
Activity/N	1 2	1 0	0 1	0 3

cytoplasmic fraction is approximately the same as the activity in the total disintegrated cell, calculated per unit of nitrogen. As the cytoplasmic fraction is a clear liquid, it is easy to handle and suitable for further purification. Table II shows that it contains comparatively large amounts of pentose nucleic acids, as determined by a modification of Dische's cysteine reactions (Brody, 1953).

Table II

ANALYSES OF THE CYTOPLASMIC EXTRACT

Extr.	*N* mg/ml	*P* mg/ml.	*Pentose nucleic acid P* mg/ml	*Schiff aldehyde reaction*	*PNAP/P*. 100
34	0·0206	0·0040	0 00139	(−)	35
36	0 0676	0 0085	0 00268	(−)	32

For further studies of the cytoplasmic fraction the method of absorption on silica or similar substances looked promising (Pontén, 1954). Table III shows an example of absorption and elution with NaCl and distilled water. A gradient of pentose nucleic acid was obtained.

Table III

FRACTIONATION ON SILICA COLUMN (PONTÉN)

	Amount ml	*Time of flow* min	µg/*PNAP*/ ml	mg *N*/ml	µg /*PNAP* / mg *N*
Original cpl-extr.	40	—	22	0 712	31
Fraction I	42	30	8	0 430	19
Fraction II (Phys. NaCl)	20	30	6·2	0 199	31
Fraction III (Phys NaCl)	24	30	1 1	0 017	65
Fraction IV (aq dest)	40	30	1 6	0·016	100

Table IV presents activity titrations of these different fractions. The activity per unit nitrogen shows an increase of about ten times compared to the original cytoplasmic extract.

Table IV

ACTIVITY-TITRATION OF THE FRACTIONS DILUTED TO EQUAL AMOUNT NITROGEN PER ML. ($1 \cdot 7 \times 10^{-2}$ MG)

	µg /*PNAP*/ ml	$1 \cdot 7 \times 10^{-2}$ mg *N*/ml	$N \times 10^{-3}$	$N \times 10^{-4}$
Cpl orig.	52	2/4	0/3	0/5
Fraction I	32	1/5	0/5	0/5
Fraction II	52	3/4	1/5	0/5
Fraction III	110	2/4	2/4	0/5
Fraction IV	160	3/5	4/5	0/5

Simultaneously, the pentose nucleic acids are concentrated three times.

I should also like to mention the fractionation of the structural constituents of the cytoplasm by differential centrifuging in sucrose media. The cells of fully developed leukæmia showed a fairly equal distribution of the virus activity between the "mitochondria" and the "microsomes," whereas the supernatant contained relatively small amounts of activity. These results are not yet unequivocal and further studies are needed.

Finally I want to stress that results from isolation in bulk of particular cellular constituents have to be regarded with great care and criticism when discussing the topographical relationships in the single cell. It seems to me, however, that work along the above-mentioned lines might offer some possibilities for studying the interplay between cellular and virus metabolism.

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DISCUSSION

[illegible]

cells

DANIELLI I would have thought that redistribution was certain.

[illegible]

chondrial fraction which cannot be freed from the virus, whereas in other fractions of the cells he doesn't find the virus But if he takes the

brains when the animals start to die, then he can wash the mitochondria free from the virus, and he finds it in the microsome fraction.

DANIELLI. If that is your trend of thought, I should like to draw your attention to the fact that there is a great deal of work showing that if the mitochondria come from really healthy cells, and are prepared under optimal conditions, one can secure dramatically rapid redistribution of substances during the process of isolation. The simplest example of this is that the mitochondria of muscle cells will take up from the cytoplasm, during the process of isolation of the mitochondria, the whole of the calcium from the muscle. Now if the mitochondria suffer any slight damage, as may happen due to an imperfect isolation procedure, or due to an unhealthy condition of the cell before the isolation procedure begins, then this type of rapid transfer of activity of a substance or of a particle may be completely inhibited. It seemed to me that since in a procedure like this you have a cell which is to some degree in a pathological state, the mitochondria under different physiological conditions would suffer different degrees of damage. You have an enormously difficult task to try and decide what was on the mitochondria before you began this fractionation procedure.

FAGRAEUS Why do you expect the mitochondria to be so much more susceptible?

DANIELLI I think all cytologists would agree that the mitochondria are among the first things to show abnormalities when a cell is getting into a bad condition.

CRAIGIE: What evidence is there that your virus is completely stable in cytoplasmic and nuclear fractions under the conditions of fractionation? Have you carried out any tests with cytoplasmic and nuclear fractions from normal cells? Is the virus more labile in one type of preparation than in the other? Or may irreversible adsorption occur?

FAGRAEUS. The two fractions were subjected to exactly the same procedures But something we haven't done is to see if the virus could be adsorbed on so-called normal tissue This has been done in the experiments on brain tissue and Theiler virus by Gard and Ostlund, where they saw that virus material could be adsorbed on normal tissue. But it is of course difficult to find a normal tissue material whose composition corresponds to leukæmia, where you have 95 per cent immature cells.

CRAIGIE. Let me put it thus way. If you keep the various fractions

[illegible]

cell material as a control, because there were not enough erythroblasts I think if you bleed controls a long time, you get about 80 per cent

erythroblasts in the bone marrow. I think you could make controls which would be very convincing if they were negative.

FAGRAEUS: We have not done that.

FURTH: It is a long time since I worked with fowl leukosis virus, but some of the unsolved problems are still present today. One difficulty encountered was the instability of the virus, particularly in attempts at purification as one gets away from its "natural" medium. One of the problems in titration tests is the choice of a medium which is not deleterious.

[illegible]

tunately, normal plasma also contains macromolecular material

[illegible]

body were introduced into the complex host, one might be able to visualize the site of the virus by radioautography. It is not inconceivable that with concentrated virus one could get serological reactions analogous to those obtained with bacteria The virus is fairly large, isn't it, about 50 micra?

[illegible]

an animal you can cause adsorption on normal material or only on the leukæmic material

WAYMOUTH Dr. Thorell said he used Tyrode to separate the cell components. I should have thought that for obtaining cytoplasmic fractions of good morphological appearance, if nothing else, the use of Tyrode would be most objectionable. Is there any objection to the use of sucrose? Is the virus less stable in the presence of sucrose?

THORELL: We have no fundamental objections to the use of sucrose, but it is difficult to wash away and therefore may interfere with the micro-determination of the nucleic acids And we are not only interested

in the distribution and structure of the morphological components of the cytoplasm. One of our main intentions was to get some fractions from the cells which we can study metabolically with isotope methods.

DARCEL: I am very interested in the numbers that you have used in your titration techniques, Dr. Fagraeus.

FAGRAEUS: There are five to six in each group. If you use this Reed and Muench estimation technique, you accumulate your material, positive as well as negative, and you measure your titre on a larger total number of animals than if you make a direct interpolation.

DARCEL: What sort of error is involved in that titration on limited numbers?

FAGRAEUS. If we consider one error, that is variation in resistance of the test animals, you can rule that out if you get a titration curve like the one I showed in the last table (Table III). When you have such a variation, and you consider your material homogeneous then you get a titration curve, say in the ten different dilution steps, such as 80, 60, 50, 30, 20

DARCEL: Is there any decrease in stability of the virus in the higher dilutions?

FAGRAEUS: I don't think so As diluent we have added 10 per cent normal rabbit serum—it was a control for a neutralization test—and then in the negative test we added negative serum, the same amount as we had positive serum, to get the neutralization index for that serum.

DARCEL. Do you find the latent period a less reliable index of activity of the different doses?

FAGRAEUS In this way we get a certain relationship, as I showed in the last table (Table III). If you can get a direct figure as we do here, it is easier than to take the incubation time. There is, however, a persistent trend towards longer incubation time with less virus.

THORELL I should like to add that we had no spontaneous leukæmia in this strain of chickens—it is a strain which has been inbred for ten years

ISRAELS I am not very clear whether you are trying to find the nature of the fowl virus, or what the virus does to the cell. This is a disease which is transmissible by cell-free filtrates. Why then are you

important. I understand that what really kills off the fowl in large numbers is a lymphatic leukæmia which is not transmissible.

THORELL. We are most interested in how the virus affects cell metabolism. I think that making preparations of the different cellular constituents is one way in which we can study the changes in metabolism induced by the virus.

FAGRAEUS I can't see any objection to the fact that if your team [illegible]

ENGELBRETH-HOLM. I think the main thing, which we have almost forgotten in the course of the discussion, is that it has been shown, in a different way from previous work, that the virus is found in the cytoplasm, and not in the nucleus. We must also remember that this work [illegible]

FAGRAEUS. No. [illegible]

the cytoplasmic fraction when you smash the cell up. That is something quite different [illegible]

virus is synthesized

FAGRAEUS I know that method of Coons quite well It has been applied to antigens of other viruses, for instance mumps virus, and they are always found in the cytoplasm. So far nobody has with certainty found any virus in the nucleus But the difficulty in our case was to

get a pure antivirus serum. That is what I am really interested in now, why I am trying adsorption on the red blood cells. But I agree with you, it is absolutely the best way. So far the methods of tracing a specific material like virus, for instance the X-ray method, and even the electron microscope and ultraviolet methods, fail because you cannot tell if the absorbing material is a virus; it is only with immunological methods that this is possible.

SOME EXPERIMENTAL OBSERVATIONS ON A STRAIN OF TRANSMISSIBLE ALEUKÆMIC LEUKÆMIA (RPL 12 LYMPHOID TUMOUR)

C. LE Q. DARCEL and G. NEGRONI

Introduction and Pathology

THE spontaneous leukotic diseases of the fowl most frequently encountered are believed to be lymphoid in origin. Although a few transplantable tumour strains have been established from lymphomatosis (Pentimalli, 1941; Olson, 1941; Burmester, 1952) the more frequent failures have been emphasized by Engelbreth-Holm (1942).

The incidence of spontaneous lymphomatosis in this country, as in the United States, is high, and the problem in its practical aspects has been under study for a number of years. In addition to field studies of this disease certain aspects of the growth and pathology of the RPL strain of Burmester have been studied (Darcel, 1953).

Following the subcutaneous inoculation of chicks with RPL tumour material, one of the most striking features observed was the frequency and rapidity with which extensive tumour cell infiltration of the liver occurred in the absence of any significant rise in the level of immature cells in the circulating blood. Blood from these chicks can induce the formation of local tumours when inoculated subcutaneously into others, a feature not observed following the inoculation of cell-free extracts of these tumours (Burmester, 1952) This suggests that tumour cells are present in the blood in numbers too few to be recognized in normal differential counts The possibility that a circulating oncogenic virus might also be involved could not be excluded, and studies on the tumour-inducing properties of the blood were made and will be discussed in this paper.

The tumour used in most of the experiments to be described was strain RPL 12 (Olson's tumour). This strain was obtained from a spontaneous case of "lymphomatosis" (Olson, 1941) and has now been passaged almost 400 times through inoculation of chicks.

Following the subcutaneous inoculation of young chicks with tumour RPL 12, a tumour develops at the site of injection after four to eighteen days, depending on the number of viable tumour cells inoculated and the rate of microscopic growth (Darcel, 1952). Death of the chicks usually occurs two to three days after the appearance of the tumour, with marked tumour infiltration of the liver, the normal architecture of which is destroyed. In early stages of growth in the liver the majority of cells lie immediately beneath the endothelial lining of the sinusoids (Fig. 1). Through the courtesy of Dr. Burmester we have had an opportunity of examining sections taken from early passages of some of the RPL tumour strains, including a section of the liver from the case of spontaneous lymphomatosis from which the apparently similar strain RPL 16 was established. In the section, tumour nodules were seen, but there was an associated leukæmia, many tumour cells being observed within the hepatic sinusoids. In a liver section from the fifth passage of this tumour, however, the cells showed a similar distribution to that seen in liver sections from RPL 12

In chicks inoculated with strain RPL 12, tumour cell infiltrations are seen far less frequently in organs other than the liver The spleen and bone marrow are rarely involved; this is in contrast to the usual replacement of these last two structures in chicks inoculated with strains of erythroblastosis. Subcutaneous tumour growth is first seen as a thin layer of tumour cells lying between the connective tissue, and there is little evidence of any stromal reaction. Following the rapid growth of the tumour, there is a great tendency for extension to the pectoral muscle Invasion of the larger blood vessels by tumour cells is often seen. Growth of cells along what appear to be lymphatics is also occasionally observed.

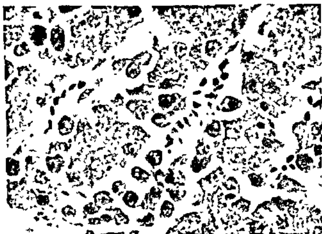


Fig 1 Section of liver from chick inoculated with strain RPL 12, showing the location of the majority of the tumour cells immediately outside the hepatic sinusoids Hæmatoxylin and eosin

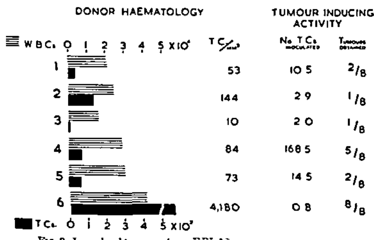
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activity are shown with the corresponding number of tumour cells estimated for each sample in Fig. 3. The results suggest that between 1 and 200 cells can result in a tumour. The fact that local tumours appeared in chicks in all positive dilutions following subcutaneous inoculation suggests that these tumours



developed from inoculated tumour cells, and not as the result of infection with a virus-like agent.

The number of tumour cells required to induce tumour formation is probably smaller than the results indicate—since other experimental data suggest that the simple dextrose or physiological saline solutions used in our experiments may be incompatible with maximal survival of the cells in the higher dilutions

In the last donor chick a true leukæmic picture was observed This has also been seen in a very few other chicks

the disease have been undertaken. Chicks were inoculated with frozen tumour mince of known activity and groups of these chicks were bled at regular intervals after inoculation. Citrated cardiac blood from each group was pooled and then inoculated without further dilution in 0·2 ml. amounts into recipient chicks. The results of these inoculations are shown

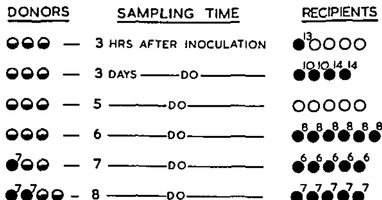


Fig 2 Lymphoid tumour strain RPL 12 —tumour-inducing activity of cardiac blood from chicks inoculated with this strain.

in Fig. 2. This shows that blood taken at nearly all times after inoculation was capable of producing tumours.

Preliminary attempts have been made to correlate tumour-inducing activity with the number of tumour cells detectable in the blood. Failure to recognize significant numbers of tumour cells in differential counts based on 200 cells led to an extension of the count to 1600 cells based on May-Grunwald and Giemsa stained smears. Individual chicks were bled from the wing vein, and the blood used for titration for tumour activity. The results of the titration for tumour-inducing

is important in view of the claim (Weisberger *et al.*, 1951) that the lungs in man and animals rapidly remove transfused normal and leukæmic leucocytes from the circulation. Although the cytological evidence suggests that the RPL strains are more closely related to erythroblastosis than the term "lymphoid" would suggest, there is an important difference between them, namely the ease with which it is possible to obtain active cell-free preparations from erythroblastosis. The question naturally arises whether it is the presence or absence of virus that is responsible for the difference in pathological features in these two strains.

The cell-free tumour-inducing agents observed by Burmester and his associates in strain RPL 12 (Burmester, 1952) are more reminiscent of the Bittner agent than an oncogenic virus of the Rous type, in that their activity and the tumours induced are similar to those obtained following the inoculation of young chicks with extracts of normal chick embryo and other tissue. It is improbable that they play any major role in the extremely rapid cellular growth of strain RPL 12.

Failure to develop tumour strains from "lymphomatosis" in the hen has been the usual experience. In view of our findings the question of whether Burmester's successes are in any way related to a high flock incidence of aleukæmic erythroblastosis, resembling visceral lymphomatosis in its gross pathology, would seem to merit consideration. This suggests that a valuable contribution could be made if systematic surveys of spontaneous neoplasia in fowls were to be undertaken with a view to correlating histological, cytological and virus transmission studies

Summary

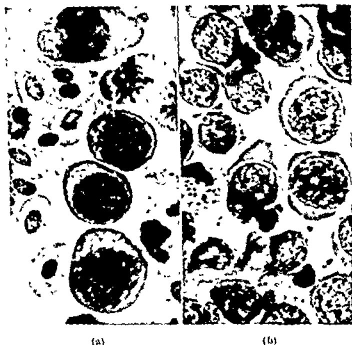
Observations on transplantable fowl tumour strains (RPL 12 and RPL 16) previously designated "lymphoma" but thought to be an aleukæmic variant of erythroblastosis, are described. They differ from typical erythroblastosis not only in the almost invariable absence of leukæmia but

inoculated with this strain. Since blood films prepared from these chicks present features identical to those seen in blood films from chicks with typical erythroblastosis, this is regarded as supplementary evidence that these strains are aleukæmic variants of erythroblastosis.

Discussion

In spite of the similarity of the cytological features of the cells of strain RPL 12 and erythroblastosis, there are differences in the gross pathology which remain to be interpreted. That the former disease is aleukæmic, with extravascular distribution of tumour cells, and the latter disease leukæmic, is of considerable interest. A comparative study of these two diseases might have a bearing on the problems of the liberation of tumour cells into the blood. Our observations of the change in type of growth between the spontaneous and later passages of RPL 16 already mentioned probably are significant in this connection. It is possible that the method of inoculation was responsible, namely repeated subcutaneous inoculation, instead of intravenously as is usually practised with erythroblastosis If this is so, attempts to develop typical erythroblastosis from these strains by choice of suitable donors, should finally meet with success.

The reasons for the aleukæmic characteristic of strain RPL 12 have not yet been elucidated. In the experiment described in which chicks were inoculated with tumour material at the extreme tip of one wing, it was not possible to demonstrate a higher level of tumour cells in blood from the wing vein of the inoculated side compared with the uninoculated side. This type of experiment must be extended to include titrations for tumour-inducing activity, since these may reflect more accurately than counts the number of viable tumour cells present. Failure to obtain any significant difference in activity between blood samples taken during the initial stages of growth of the primary tumour would indicate that the lungs and other organs are not removing a high percentage of the tumour cells from the circulation. This



(a) (b)

FIG 4 (a) Impression smear from liver (RPL 12) showing proerythroblast cells (type I) May-Grunwald and Giemsa (b) Impression smear from liver (erythroblastosis) May-Grunwald and Giemsa Compare with (a)

also in the organ selectivity of the tumour cells. Tumour-inducing activity of the blood can be demonstrated at nearly all stages after inoculation and appears to be related to the number of circulating tumour cells. The number of cells required to induce a tumour is small, probably less than one hundred.

I
t
and others.

APPENDIX

Attempts to correlate tumour-inducing activity with the number of tumour cells in the blood would be meaningless unless the cells can be recognized with accuracy. This, and the need to determine the position of these cells in relation to the classification of the cells of the lympho-hæmopoietic system, were the main reasons why a study of the cytology of these tumour cells was made.

Three main types of cells have been observed in the present study. Type I (Fig. 4a). These are 12 μ in mean diameter, and ovoid, with nuclear cytoplasmic ratio in favour of the nucleus. The cytoplasm is deeply basophilic, often with a *paler zone next to the nucleus. There are no granules in the* cytoplasm but vacuoles are often observed. The nucleus has a well-defined nuclear membrane and shows a very fine structure rich in chromatin, uniformly distributed in a thread-like pattern interrupted only where nucleoli, more or less prominent, are seen. Type II (Fig 5). These are approximately 18 μ in mean diameter, but are very difficult to measure because of the irregular and ill-defined cytoplasmic membrane. The cytoplasm is less basophilic than in the first type but is free of granules. The nucleus also shows a well-defined membrane but is less rich in chromatin and has a reticular structure. Type III (Fig. 6): These are very large cells with a mean diameter of 21 μ. The cytoplasmic characters are

similar to those of Type I. The nucleus, however, is very large and spherical, with an ill-defined structure. The chromatin is relatively scarce.

With regard to the nature of these cells, we feel certain that Type I must be classified as pro-erythroblastic in accordance with the classification of Ferrata and Storti (1948). The deep basophilia of the cytoplasm of these cells is typical only of the immature red cells of the series and is a differentiating feature from lymphoblasts The latter are usually slightly smaller, with a very narrow rim of uniformly staining and slightly basophilic cytoplasm Another difference is the tendency of the chromatin of lymphoblasts to form clumps attached to the surface of the nuclear membrane in preparations stained with May-Grunwald and Giemsa As a result the nucleus appears almost empty and very poor in chromatin, with the usually single nucleolus becoming very prominent.

Whilst Type I is easily classified as pro-erythroblastic, *the nature of Types II and III remains obscure. Although* different morphologically, both are obviously related to Type I, but whether they are to be regarded as degenerative or more or less differentiated forms of these cells is an open question.

However, not one of these three types is suggestive of an atypical cell of the lymphoid series. For this reason we consider that it is misleading to designate transplantable tumour RPL 12 as a "lymphoid" tumour, although on the basis of the macroscopic appearance at autopsy in the original bird, such a terminology might appear justified. In this connection it should be noted that Engelbreth-Holm (1942) has observed that erythroblastomata are often encountered in association with erythroblastosis A difference of mode of growth of the tumour cells in present passages from those of typical erythroblastosis now exists, but we do not regard this extravascular growth as adequate evidence of its lymphoid nature. The intravascular growth in the original bird from which Strain RPL 12 is derived and the present extravascular growth

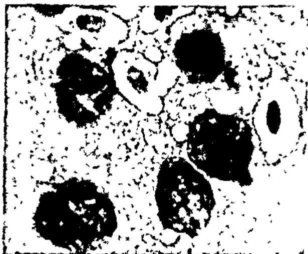


Fig 5. Impression smear from subcutaneous tumour (RPL 12) showing stem-cells (type II) May-Grunwald and Giemsa.

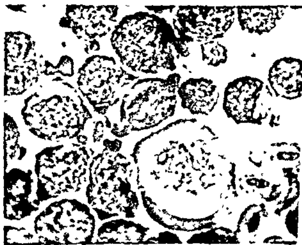


Fig 6 Impression smear from liver (RPL 12) showing one giant cell (type III) May-Grunwald and Giemsa

similar to those of Type I. The nucleus, however, is very large and spherical, with an ill-defined structure. The chromatin is relatively scarce.

With regard to the nature of these cells, we feel certain that Type I must be classified as pro-erythroblastic in accordance with the classification of Ferrata and Storti (1948). The deep basophilia of the cytoplasm of these cells is typical only of the immature red cells of the series and is a differentiating feature from lymphoblasts. The latter are usually slightly smaller, with a very narrow rim of uniformly staining and slightly basophilic cytoplasm. Another difference is the tendency of the chromatin of lymphoblasts to form clumps attached to the surface of the nuclear membrane in preparations stained with May-Grunwald and Giemsa. As a result the nucleus appears almost empty and very poor in chromatin, with the usually single nucleolus becoming very prominent.

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with erythroblastosis A difference of mode of growth of the tumour cells in present passages from those of typical erythroblastosis now exists, but we do not regard this extravascular growth as adequate evidence of its lymphoid nature. The intravascular growth in the original bird from which Strain RPL 12 is derived and the present extravascular growth

has already been described in the main paper. We therefore suggest the name "erythroblastoma" for this condition and regard this as an aleukæmic form of erythroblastosis. A comparative study of the biological behaviour of these strains and erythroblastosis is in progress.

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DISCUSSION

Craigie. The chief reason for choosing the RPL strains for special study was that these so-called lymphoid tumours seem to provide experimental material for the development of techniques to achieve greater certainty regarding the presence or absence of an oncogenic agent which might elude conventional techniques for the demonstration of virus or virus-like agents So far, the evidence is that the RPL strains have no associated oncogenic virus having the short latent period characteristic of erythroblastosis virus, the existence of a virus-like

hosts in which the disease had progressed from a dependent or conditional leukæmia to the emergence of autonomous properties, thus

rather a very young type of leukosis and some of the pictures resemble closely the ones I shall show which are derived from Murray-Begg's endothelioma.

Our strain of leukosis, which at the beginning was a pure erythroblastosis, changed gradually and became a hæmocytoblastic type, with participation of the reticulo-endothelial cells in bone-marrow, liver, spleen, and it was at that time that the sarcomas appeared So, it seems to me that this leukæmia is not of a conventional erythroblastic type, I would say it is probably a hæmocytoblastic form.

[illegible]

the tumour cells are seen in the spleen they do not seem to have any relation to the normal lymphatic tissue of the organ.

Apart from any other considerations, I feel strongly that it is possible to recognize a cell of the red series easily from the first stage of maturation onwards.

FURTH I too was puzzled by the pictures. It is one matter to identify a cell and another to know about the inter-relationship of various cell types Lymphocytes are motile cells; erythroblasts are not, and there are many other cytological differences between them. I have never seen a transition from the lymphoid to the erythrogenic type Lymphomatosis often does not involve the bone marrow, and there is no anæmia with it, the lymphoid infiltrations are usually extra- and not intravascular, as in erythroleukosis However, in many instances there is bone marrow involvement with lymphoid leukæmia, and secondary anæmia with secondary erythroblastosis when both cell types are [illegible]

whether the perivascular space you showed is analogous to the lymphatics of man (Disse's space) In erythroblastosis the intravascular accumulation of erythroblasts is a characteristic feature, while the lymphocytes tend to get out of vessels. The classical picture of lymphomatosis is that of perivascular infiltration, with portal infiltration in the liver and infrequent and variable intravascular accumulation of lymphoid cells

The terms "leukæmia" and "aleukæmia" used by hæmatologists are almost meaningless to us As you have shown, the blood can transmit the aleukæmic disease. It has also been shown that mouse leukæmia

is usually preceded by an aleukæmic form during which lymphocytes circulating in the blood in very small numbers can transmit the disease. True aleukæmic lymphoma is also rare in man I recall one patient with the clinical and post-mortem diagnosis of lymphosarcoma, in whom review of the sections showed a marked lymphostasis in the capillaries of the kidneys suggestive of "true" leukæmia. Presumably, such a lymphosarcoma would transmit the disease. There is a great deal of uncertainty about the term leukæmia. Lymphocytes from aleukæmic lymphomatosis tend to produce a predominantly aleukæmic type of

DARCEL We were very impressed by the extravascular appearance at one time, and thought that this was the essential difference between these lymphoid tumours and erythroblastosis. However, we now think that the change in type of growth between the spontaneous case in which RPL 12 originated and the fourth passage is very important. It is quite possible that the present appearance of these tumours might be the result of the technical approach. Because we do occasionally get these leukæmic

from erythroblas

donors, as Dr. F

blastosis strain.

are quite encour

than we had ever observed before by intravenous passage of blood from leukæmic chicks. The pathology in these cases is showing less tendency for the extravascular and more tendency for the intravascular distribution of cells.

MOESCHLIN I think we have to be careful in cases like this and should just speak of a blastic type of cell We have probably all seen such cases

cally.

A promising approach was made recently by Birman's group (Medical Dept , Malmo, Sweden), for the differentiation of the erythroblastic type. He showed in a case of human erythroblastosis that you can

FAGRAEUS· Yes, you can

ENGELBRETH-HOLM. There is another way perhaps. We are up against the usual problem of differentiating or classifying the stem cell, and I quite agree with Dr. Moeschlin that from purely morphological features it is next to impossible in many cases. Some thirty years ago

the icebox for about twenty-four hours before you fix it. Then the

opinions expressed so far were against viruses. I believe that it has been proved that lymphomas can also contain a virus and that there are several such viruses*. It is relatively easy to prove that a tumour has a virus and difficult to certify that it has not.

Another point is the range of tissue affinity of the viruses. Some of them affect only one type of cell and others several types of cells.

NEGRONI It was impossible to produce the disease by injecting filtrates

WITTS Dr Furth raised this point of aleukæmic and leukæmic leukæmias, suggesting that it was something inherent in the cell. I wonder whether there are any comments on that

BURCHENAL. I think all clinicians have noted that if a child with

relapse the count goes up.

JACOBSON. But of course one doesn't know the natural history of the

*[For references see Burmester, B. R. (1947) *Cancer Res*, 7, 786, Burmester, B. R. and Gentry, R. F (1954) *Cancer Res*, 14, 34, Duran-Reynals, F. (1950) *Amer. J. med*, 8, 490

disease in this latter situation. The child seen for the first time may have already passed the early leucopenic phase of the disease. A recent report by Block has stressed the appearance of a leucopenic phase in the peripheral blood and a relative aplasia in the marrow for months prior to the onset of a frank diagnosable acute leukæmia.

GORER: A lot of these acute leukæmias come in as allegedly pernicious anæmias. We had the impression that when we gave them liver they tended to become leukæmic, and I should be interested to know if other people have seen this

MOESCHLIN. It has also been seen under folic acid therapy.

OBERLING: The influence of liver extracts might be related to the question of leukæmic and aleukæmic forms which have been studied now for years in a strain of rat myelosis This strain started as a myeloid chloro-leukæmia with chloroma, the leukæmic factor disappeared in the following passages and the lesion was then transplanted for about ten years as a pure myeloma Then we tried to go back to the leukæmic type, and we were able to do this by transplanting cells into rat embryos just before birth; when the rats were eight days old they came down with leukæmia. We found that the factor involved was probably nutritional, because in adult rats too leukæmias could be obtained when the tumour-material was injected into animals fed a regime rich in wheat germ Now we are trying to find out the factor in wheat germ oil which promotes this leukæmic type.

MOESCHLIN: I think there are different reasons why one patient or mouse has leukæmic and another aleukæmic leukæmia. One reason is the location where the cells are growing. Usually in aleukæmic cases we find that the bone marrow is involved and not the glands and the spleen (see Moeschlin and Rohr, 1939, *Ergebnisse der Kinderheilkunde*, 57, 123) As soon as the spleen gets involved and enlarged, usually the patient's count starts to rise. I think this is the explanation for the type of patient that Dr. Burchenal mentioned, the aleukæmic patient whose count goes down when there is a relapse In these patients there is an [illegible]

for some cases

ENGELBRETH-HOLM I am very glad that Dr Moeschlin brought out Rohr's theory about the explanation of the aleukæmic states. I can [illegible]

Witts: Of course we still may get back to Dr. Furth's point, that what may be inherent in the cell is this tendency to colonize organs like the spleen There is no contradiction of fundamentals

Gross: I have been interested in the leukæmic and aleukæmic leukæmias in our mice, but I have no explanation as yet. In many

death.

Gorer There is another point about leukæmia and aleukæmia, and that is where you take the blood from In mice you may find that the blood from the tail vein is aleukæmic and the heart blood leukæmic.

Israels: Surely one of the answers to this problem is, as Dr Jacobson said, that in so many cases you don't know the natural history of the disease For example, if you find leukæmia by a bone marrow puncture

like these tumours They are predominantly infiltration tumours. If

different natural history.

TRANSMISSIBLE MOUSE LEUKÆMIA: BIOLOGICAL PROPERTIES OF THE MOUSE LEUKÆMIA AGENT*

LUDWIK GROSS

Leukæmia is essentially a rare disease in mammals. Yet, by pedigreed inbreeding of descendants of leukæmic mice, families of mice were eventually obtained in which very large numbers of animals of both sexes would spontaneously develop leukæmia. Furth and his co-workers (1933) developed (Cole and Furth, 1941) a strain of albino mice, designated by the symbol AK, having a remarkably high incidence of spontaneous leukæmia. We had the good fortune to receive a litter of AK mice, in November 1945, from Dr. Furth, then at Cornell University, and have raised thus far twenty-five generations of these animals by brother-to-sister mating. In our colony of AK mice, over 80 per cent of both males and females spontaneously develop leukæmia at an average age of 8 5 months.

The diagnosis of leukæmia has been made in our laboratory on the basis of typical, enlarged, pea-sized peripheral lymph nodes, a large cylindrical white and friable tumour in the mesentery, greatly, sometimes enormously, enlarged spleen and liver, and in some instances also the presence of a large tumour in the mediastinum In all instances microscopic sections were made of livers, showing infiltration with leukæmic cells. The white cell count (tail blood) varied from 30,000 to 240,000 per mm.³, with a differential count frequently showing immature white cells and a high predominance of lymphocytes on the smear. Occasionally, however, the peripheral white blood count appeared to be normal, even

*Aided, in part, by a grant from the Damon Runyon Memorial Fund for Cancer Research.

though the sacrificed mice presented a typical picture of leukæmia. The bone marrow picture was more consistently typical for leukæmia. We found, however, that the general clinical picture, such as a very large spleen and liver, pea-sized peripheral lymph nodes, a large tumour in the mesentery and in the mediastinúm, and particularly the infiltration of organs, such as liver or kidneys, with leukæmic cells (evident on microscopic examination), were more reliable in establishing the diagnosis of leukæmia than examination of the peripheral blood.

Is Mouse Leukæmia caused by a Transmissible Agent?

The question remained to be answered whether the development of spontaneous leukæmia in mice of the AK inbred line is caused by an obscure process of mutation, or whether it could be attributed to a transmissible pathogenic agent. Since the demonstration, by Ellermann and Bang (1908), that chicken lymphomatosis is caused by a filtrable agent, the possibility had to be considered that in other species also, and particularly in the mouse, leukæmia may be caused by a transmissible, cell-free agent. Attempts, however, to demonstrate the presence of such an agent in mouse leukæmia failed, until newborn mice were used for the inoculation of the leukæmic extracts (Gross, 1951*a*)

It should be emphasized that under the current experimental conditions, the presence of a submicroscopic, invisible agent, presumably responsible for the development of a tumour, or of leukæmia, can be determined only by a biological assay In the case of mouse mammary carcinoma, the presence of the mammary carcinoma agent could be determined by inoculating a biological sample, such as a tissue extract, tested for the presence of the agent, into ten- to twenty-day-old suckling infant mice of a susceptible line, free from spontaneous mammary carcinoma, in order to find out whether the inoculated animals would develop "spontaneous" mammary carcinomas after they reach middle age. It was therefore anticipated that, should mouse leukæmia also be

caused by a transmissible agent, such an agent could be detected by a similar bioassay. Such a bioassay would, therefore, consist of injecting biological samples, presumably containing the leukæmic agent, into very young mice of an inbred line known to have been hitherto free from this disease, with the purpose of determining whether the inoculated animals would develop "spontaneous" leukæmia after middle age.

We had in our laboratory mice which appeared suitable for this type of experimental tests, namely mice of either the C3H line, or of the foster-nursed, i.e. free from mammary carcinoma agent, $C3H_f$ subline. Our C3H colony has been raised, by brother-to-sister mating, from a litter obtained in July 1944 from J. J. Bittner of the University of Minnesota; this colony is now in its 29th generation. On April 2, 1949, a litter removed by Cæsarean section from a pregnant (at term) C3H female, was foster-nursed by a C57 (black) female, and from this foster-nursed C3H litter another colony has been raised in our laboratory by brother-to-sister mating, and designated by the symbol $C3H_f$; this subline is now in its eighteenth generation. Mice of our colony of this subline, being descendants of foster-nursed C3H mice, do not carry the mammary carcinoma agent. Accordingly, mammary carcinomas develop in the great majority of females in our colony of C3H mice, but only very rarely in those of the foster-nursed $C3H_f$ subline. Leukæmia, on the other hand, is extremely rare in either the C3H line, or in the $C3H_f$ subline. We have seen only four cases of spontaneous leukæmia among the 6,000 C3H, and 2,100 $C3H_f$ mice observed in this laboratory. It is true that among the C3H females of our colony many died from mammary tumours, and many other males and females were sacrificed before reaching the advanced age at which spontaneous leukæmia might have developed. We have observed, however, in our own laboratory, at least 1,000 C3H and $C3H_f$ males and females through their full life span, and can assume that the percentage of spontaneous leukæmia among these animals is extremely low, far below 0·5 per cent.

(The incidence of spontaneous leukæmia among 298 C3H *mice observed by Furth, Cole and Boon (1942)* was 0·3 per cent.)

Accordingly, mice of either the C3H line or of the foster-nursed $C3H_f$ *subline appeared eminently suitable* as test animals for the inoculation of extracts presumably containing the leukæmic agent. Newborn, suckling infant mice were used for inoculation, because in the case of mammary carcinoma, young suckling mice had been used for the biological assay, adult animals being for all practical purposes resistant to inoculation with the mammary carcinoma agent (Bittner, 1944; Andervont, 1945; Dmochowski, 1945). Whereas, however, in the case of mammary carcinoma, young mice up to twenty-one days of age could be readily infected with the mammary *tumour agent* (Bittner, 1944), *it soon became* evident that in the case of mouse leukæmia, the inoculation, in order to succeed, should take place within sixteen, and *better within twelve hours after the birth of the animal* (Gross, 1951*a* and *b*).

The tentative plan of the experiment was, therefore, as follows: extracts were to be prepared from AK mice; these extracts were to be injected into newborn, suckling C3H, or $C3H_f$ mice; the inoculated mice were then to be observed for the possible development of leukæmia

Inoculation of AK Leukæmic Cells

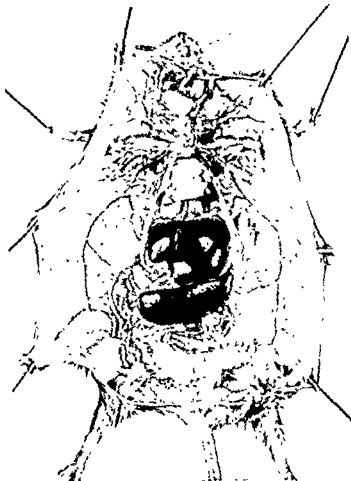
In preliminary experiments, AK leukæmic cell suspensions *of 20 per cent concentration (prepared by grinding spleen,* liver, and tumorous lymphatic glands of leukæmic AK mice with sterile physiological saline solution) were inoculated *into newborn C3H mice* It was first thought that should the AK leukæmic cells actually contain a transmissible agent, it would be preferable to inject leukæmic cells, instead of centrifuged or filtered extracts, because cell suspensions might contain a higher concentration of such an agent. It was soon observed that newborn, suckling C3H mice are highly susceptible to the implantation of AK leukæmic cells

(Gross, 1950); in most of them, leukæmic tumours developed at the site of implantation of the leukæmic cells within two to three weeks, followed usually within a week or two by a generalized leukæmia. This proved to be a transplanted AK leukæmia, however, the suckling C3H mice serving only as a medium for the growth of the implanted AK leukæmic cells. The leukæmic tumours that developed in newborn C3H mice as a result of the implantation of AK leukæmic cells, could be transplanted, by cell graft, back to the donor animals, i.e. to adult AK mice, but would not grow, except in rare instances, if transplanted to adult C3H mice (Gross, 1953*a*). The susceptibility of the suckling C3H mice to implantation of AK leukæmic cells was found to be limited to the first few days of their lives. Adult C3H mice were found to be, with rare exceptions, resistant to the implantation of large doses of AK leukæmic cells (Gross, 1950).

Inoculation of Centrifuged AK Leukæmic Extracts

In the next series of experiments, the leukæmic cell suspensions of 20 per cent concentration, prepared in the usual manner by grinding spleen, liver and lymphatic tumours of leukæmic AK mice with sterile physiological saline solution, were centrifuged at 3,000 r.p.m. at 0°C., for thirty minutes. Newborn C3H, and $C3H_f$ mice, were then inoculated with the supernatant. Of 36 mice inoculated when less than one day old, 29 (81 per cent) developed typical "spontaneous" leukæmia at an average age of 10·6 months (Gross, 1951*b*). These mice had enormous spleens and livers, pea-sized tumorous lymph nodes in the inguinal and axillary pits, and infiltration of organs, such as liver and kidneys, with leukæmic cells, evident on microscopic sections.

Since we were not convinced that centrifugation at 3,000 r.p.m. was sufficient to sediment all leukæmic cells, a second series of experiments was carried out in which the AK leukæmic extracts of 20 per cent concentration, prepared in the usual manner, were first centrifuged at 3,000 r.p.m. at 0°C., for fifteen minutes, and the supernatant was again



[To face page 80

centrifuged at 9,500 r.p.m. (7,000×g), at 0°C., for ten to fifteen minutes. Newborn C3H$_f$ or C3H mice were then inoculated subcutaneously with the final supernatant, at an average age of fifteen hours. Each newborn litter of mice was divided as follows: one part of the litter was injected with the fresh supernatant; another part, serving as control, was injected with a supernatant that had been heated to 65°C. for thirty minutes. For the purpose of identification, the infant mice injected with the heated supernatant had their tails tied, and cut, at half length. Immediately following inoculation, the skin punctures were dried off gently with a sterile sponge, and sealed with a drop of collodion. Of the 41 C3H and C3H$_f$ mice that were inoculated with the fresh supernatant, 19 developed typical leukæmia (46 per cent) at an average age of 5·2 months (Gross, 1953*a*). Twenty-six litter mates that had been inoculated with the heated extracts remained in good health (Table I). Leukæmia developing

Table I

RESULTS OF INOCULATION OF CENTRIFUGED (9,500 RPM) AK-LEUKÆMIA EXTRACTS INTO NEWBORN C3H OR C3H$_f$ MICE

Fresh supernatant				*Heated (65°C ½ hr) supernatant*	
Average age at inoc (hr)	*No of Mice inoc*	*No of Mice Devel Leuk*	*Average age when Leuk Devel (months)*	*No of Mice inoc*	*No of Mice Devel Leuk*
15	41	19	5 2	26	0

in the C3H mice that had been inoculated with the fresh supernatant was readily transplantable by cell graft to adult mice of the C3H line, but only exceptionally to those of the AK line (Gross, 1953*a*). These transplantation results suggest that the leukæmia which developed in C3H mice as a result of inoculation with the centrifuged AK leukæmic extracts was a "C3H leukæmia" due to the pathogenic action of a cell-free agent on the cells of the host. Leukæmic cells developing in such hosts would, therefore, be of the host's

genetic origin and should therefore be readily transplantable to individuals of the host's inbred line, i.e. to adult C3H mice. Had these mice developed a "transplanted" AK leukæmia, possibly as a result of inadvertent implantation of some stray leukæmic cells accidentally floating in the supernatant, a subsequent attempt to transfer, by cell graft, the resulting leukæmia to adult mice of the C3H line would most likely not have succeeded.

The reason why the C3H mice that had been inoculated with the 9,500 r.p.m. (7,000×*g*) AK leukæmic supernatant developed leukæmia earlier (at an average age of 5·2 months) than those inoculated with the 3,000 r.p.m. supernatant (average age 10·6 months) is not quite clear. It is possible, however, that the centrifugation at the higher speed, i.e. at 9,500 r.p.m., sedimented an inhibitor, thus releasing a more active agent present in the supernatant. In any event, since this observation was made, centrifugation of AK leukæmic extracts at 9,500 r.p.m. has become in our laboratory a standard procedure in experiments in which the AK leukæmic agent is to be inoculated into C3H mice.

Inoculation of Filtered (Seitz) AK Leukæmic Extracts

In order to eliminate any doubts concerning the possibility of an accidental implantation of a few leukæmic cells when using centrifuged extracts for inoculation, a series of experiments was carried out in which the AK leukæmic extracts of 20 per cent concentration, prepared in the usual manner by grinding livers, spleens, and lymphatic tumours from AK leukæmic mice with sterile physiological saline solution, were centrifuged at 3,000 r.p.m., for fifteen minutes, and the supernatant was then passed through Seitz filter pads (S-1), under a vacuum pressure of approximately 20 mm. of mercury, using a water aspirator. Immediately after the filtration of the leukæmic extracts, all filters were tested with, and found to be impervious to, *E. coli*. In 28 experiments, requiring 28 individual filtrations, 111 newborn $C3H_f$ or C3H mice were inoculated subcutaneously with the filtered leukæmic

extracts at an average age of nine hours. As a result, 30 of the 111 mice (32 per cent) developed "spontaneous" leukæmia at an average age of fourteen months (Table II).

Table II

RESULTS OF INOCULATION OF FILTERED (SEITZ, S-1) AK LEUKÆMIC EXTRACTS INTO NEWBORN C3H OR C3H$_f$ MICE

Fresh filtrate			
Average age at inoc (hours)	*No of Mice inoc*	*No of Mice Devel Leuk*	*Average age when Leuk Devel (months)*
9	111	30	14

Inoculation of Filtered (Berkefeld-N) AK Leukæmic Extracts

In another series of experiments, the AK leukæmic extracts of 20 per cent concentration, prepared in the usual manner, were centrifuged first at 3,000 r.p.m. for fifteen minutes; the supernatant was then removed, and centrifuged at 9,500 r.p.m. (7,000 × g) for periods of time varying from one to ten minutes (average time 4·5 minutes). The final supernatant was then passed through Berkefeld filter candles, porosity N, under a vacuum pressure varying from 20 to 25 mm of mercury. Immediately after the filtration, all filter candles were tested with, and found to be impervious to, *E. coli* In 30 experiments, requiring 27 individual filtrations, 133 newborn C3H$_f$ or C3H mice were inoculated subcutaneously with the fresh filtered AK leukæmic extracts at an average age of *nine* hours; as a result, 17 developed "spontaneous" leukæmia (13 per cent) at an average age of [illegible] mice in this group, [illegible] gland [illegible] s. Of [illegible] same time with heated filtered [illegible] loped leukæmia, but two of *them* developed salivary gland carcinomas at four months of age (Table III)

Table III

RESULTS OF INOCULATION OF FILTERED (BERKEFELD-N) AK LEUKÆMIC EXTRACTS INTO NEWBORN C3H OR C3H$_f$ MICE

Fresh filtrate						Heated (68°C ½ hr) filtrate			
Av age at inoc (hr)	*No of Mice inoc*	*No of Mice Devel Leuk*	*Av age when Leuk Devel (mths)*	*No of Mice Devel Sal G. CA*	*Av age when Sal G CA Devel (months)*	*No of Mice inoc*	*No of Mice Devel Leuk*	*No of Mice Devel Sal G CA*	*Av age when Sal G CA Devel (months)*
9	133	17	5 7	11	5 6	111	0	2	4

This series of experiments is still in progress, some of the inoculated mice being now only six and a half months old. It is possible that a few more mice in this experimental series will develop either leukæmia or salivary gland carcinomas.* In any event, the results thus far obtained suggest that the AK leukæmic agent can be filtered through the Berkefeld-N candles, and that it does not resist heating to 68°C. for thirty minutes The fact that some of the mice that had been inoculated with the filtered extracts developed salivary gland tumours, instead of leukæmia, is puzzling (Gross, 1953*b*); these tumours have never been observed in untreated C3H or C3H$_f$ mice among the animals of our colony; they are extremely rare in mice of any strains, and only very few have been described in mice of strains A or C (Lippincott, Edwards, Grady and Stewart, 1942).

The observation that two out of 111 C3H mice that had been inoculated with heated (68°C) filtered AK leukæmic extracts developed salivary gland tumours, could be explained by assuming that the filtered AK leukæmic extract

*ADDENDUM As this manuscript goes to press (April, 1954), among the additional mouse developed salivary gland carcinoma (making a total of three in this group)

requires heating for thirty minutes to a temperature higher than 68°C. for complete inactivation. On the other hand, the possibility should also be considered that the control litter-mates that had been inoculated with the heated extracts, became infected by contact of their skin wounds with the fresh leukæmic extracts, possibly oozing from skin puncture wounds of their litter-mates, sharing the same nest and nursed by the same mother.

The possibility of such an accidental wound infection, through contact, should be taken into consideration particularly in view of the results recently obtained in experiments dealing with dosage of the AK leukæmic extracts. These experiments are still in progress, and will be reported at a later time, it is already evident, however, that the AK leukæmic extracts retain their pathogenic potency even in dilutions up to 1 : 100,000. Thus, following inoculation of 0·1 ml of a 1 . 100,000 dilution of the centrifuged (9,500 r.p.m.) AK leukæmic extract into newborn C3H mice, typical salivary gland carcinomas developed in some of the inoculated mice (Gross, 1954) Since such small quantities of the AK leukæmic extracts were sufficient to cause the development of these tumours in C3H mice, it appears conceivable that some of the litter-mate control suckling mice, sharing the same nest with their siblings (which had been simultaneously inoculated with fresh, active extracts) might have become accidentally infected by contact. The point of the possible entry of the oncogenic agent might have been either the skin puncture, incidental to the introduction of the needle at subcutaneous inoculation of the extract, or the wound of the tail, since the control litter-mates had their tails cut immediately after birth for the purpose of identification

Inoculation of Filtered (Selas) AK Leukæmic Extracts

In a third series of filtration experiments, the AK leukæmic extracts of 20 per cent concentration, prepared in the usual manner, were centrifuged first at 3,000 r.p m. at 0°C. for

fifteen minutes; the supernatant was removed, and again centrifuged at 9,500 r.p.m., at 0°C., for five to ten minutes The final supernatant was then passed through Selas microporous porcelain filter candles (porosity 03, and in a few experiments those of porosity 02). Immediately after the filtration of the leukæmic extracts, all filter candles were tested with, and found to be impervious to, *E. coli.*

In 33 experiments, requiring 16 individual filtrations, a total of 141 newborn C3H, and C3H mice was inoculated subcutaneously with the fresh filtered AK leukæmic extracts at an average age of twelve hours. Each newborn litter was split at the time of inoculation: one part of the litter was inoculated with the fresh filtered extracts, and another with an extract which had been heated to 68°C. for thirty minutes. *Of the 84 mice that had been inoculated with the fresh filtered* extracts, 18 developed typical leukæmia (21 per cent) at an average age of 6·9 months, and 17 additional mice (20 per cent) developed, instead of leukæmia, bilateral salivary gland carcinomas at an average age of 3·9 months (Figs. 3 and 4).* (A summary of this series of experiments dealing with the filtration of the AK leukæmic extracts through Selas filter candles was published in one of our previous papers (Gross, 1953*b*); the data presented in this report are adjusted, however, because additional mice developed either leukæmia or salivary gland carcinomas, as indicated in Table IV.) No leukæmia developed among the 57 control litter mates that had been inoculated simultaneously with the heated filtered extracts; one mouse in this control group, however, developed salivary gland carcinoma at the age of eight months (Table IV).

group)

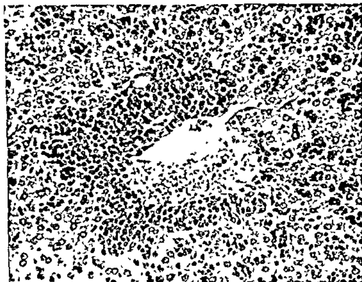


Fig 2 Photomicrograph of liver of a C3H female mouse with "spontaneous" leukæmia This mouse was inoculated when less than five hours old, with a filtered (Berkefeld, N) AK leukæmic extract, and developed leukæmia at eight months of age Typical infiltration with leukæmic cells, particularly prominent around the larger blood vessels (H & E ×200)

[To face page 86



leukæmia, carcinoma of the salivary (parotid) gland.

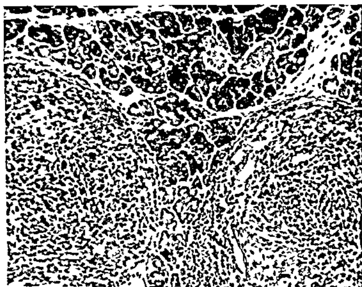




Table IV*

RESULTS OF INOCULATION OF FILTERED (SELAS) AK LEUKÆMIC EXTRACTS INTO NEWBORN (< TWELVE HOURS OLD) C3H OR C3Hf MICE

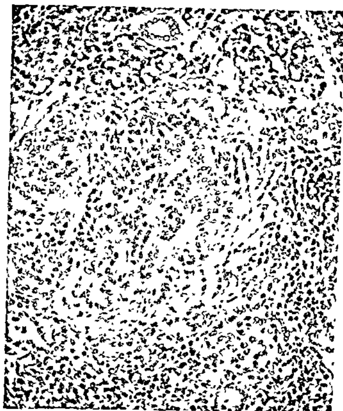
	Fresh filtrate					Heated (68°C ½ hr) filtrate			
Filter pores	No of Mice inoc	No of Mice Devel Leuk	Av age when Leuk Devel (months)	No of Mice Devel Sal G C A	Av age when Sal G C A Devel (months)	No of Mice inoc	No of Mice Devel Leuk	No of Mice Devel Sal G C A	Av age when Sal G C A Devel (months)
03	75	15	6.9	17	3.9	51	0	1	8
02	9	3	5.3	0	0	6	0	0	0
Total	84	18		17		57	0	1	

*See Addendum, p 86

As in the experiments dealing with filtration of the AK leukæmic extracts through Berkefeld candles, in the Selas series also the development of a salivary gland tumour in one of the 57 control mice that had been inoculated with the heated extracts could have been caused either by the fact that the AK leukæmic extracts may require a slightly higher temperature for complete inactivation, or because this mouse, sharing the nest since birth with siblings that had been inoculated with fresh, active extracts, might have developed a salivary gland tumour because of an accidental contact infection through the skin puncture wound.

Salivary Gland Tumours Developing in C3H Mice following Inoculation of Filtered AK Leukæmic Extracts

The salivary gland tumours that developed in some of the C3H mice that had been inoculated with the filtered (Berkefeld or Selas) AK leukæmic extracts, appeared in some of the animals quite early, sometimes at three or three and a half months of age. They developed usually on both sides of the neck, and increased very slowly in size, forming subcutaneous grape-like clusters composed of small, individual tumours. Eventually, however, they formed enormous confluent tumours surrounding the necks of the animals in a grotesque



common in these animals, it would follow that in mice of the AK line the leukæmic agent interferes with the salivary gland tumour agent, and/or that the AK mice are not susceptible to the pathogenic action of the tumour agent, even though they carry it (Gross, 1953*b* and *d*).

Preservation of the Salivary Gland Tumour Agent by Dry-freezing

Preliminary experiments demonstrated that lyophilized AK leukæmic extracts could be kept at 4°C in sealed ampoules for thirteen months, retaining their ability to reproduce salivary gland tumours following inoculation of such extracts into newborn C3H mice (Gross, 1953*d*). Whether the AK leukæmic agent could also be preserved by dry-freezing has not yet been determined; these experiments are now in progress, and will be reported at a later date.

Presence of the Leukæmic and Tumour Agents in Normal AK Embryos

Since leukæmia develops "spontaneously" in successive generations of mice of the AK line, and since it was found to be caused by a filtrable agent, it was reasonable to assume that such an agent is transmitted naturally from one generation to another. Unlike the mammary carcinoma agent, however, the mouse leukæmia agent was found to be transmitted from one generation to another not through the milk of nursing mothers, but through the embryos (Gross, 1951*a* and 1952), i.e. most probably directly through the germinal cells (Gross, 1953*c*). The possibility of a transmission of the leukæmic agent through the milk was excluded in an experiment in which a litter was removed, at term, by Cæsarean section from a healthy AK female, and was then nursed by a foster mother of the C57 (black) line (free from spontaneous leukæmia); of the three foster-nursed AK mice that survived, two developed "spontaneous" leukæmia. The three foster-nursed mice had 13 offspring, and 11 of these died from leukæmia at 8 1 months of age (Gross, 1952). Previous

manner. Quite frequently, secondary tumours appeared later in the auxillary or inguinal pits of these mice, and occasionally also in the abdominal cavity. The lymphatic glands, however, remained normal. The peripheral blood and bone marrow showed no pathological changes. The liver and other internal organs also remained normal, showing no trace of leukæmic infiltration so typical in the case of leukæmia: the spleen was occasionally slightly enlarged, but far from the size observed in leukæmia.

The neck tumours were found to arise in multiple foci in the salivary glands; in the animals thus far examined, the parotid gland appeared to be the site of the primary tumours. Microscopically these tumours could be classified as anaplastic carcinomas; some of the secondary inguinal or axillary tumours in these animals, had, however, a microscopic structure resembling a sarcoma; in a few instances even the primary salivary tumours had an appearance resembling either a carcinoma or a sarcoma, these different patterns occurring in the same salivary tumour depending on the part of the tumour examined (Gross, 1953*b*). The salivary gland tumours could be transplanted subcutaneously, by cell implantation, into mice of the C3H line, but did not grow when implanted into adult AK mice. In the C3H mice, on the other hand, they grew readily at the site of implantation, infiltrating the surrounding tissues, but remaining localized, and eventually forming enormous subcutaneous tumours. We have never observed any evidence of leukæmia in mice that had been inoculated with the salivary gland tumour cell suspensions

Theoretically, it could be assumed that the leukæmic agent recovered from AK leukæmic extracts could produce, following inoculation into newborn C3H or C3H*f* mice, either leukæmia or salivary gland tumours. It appears more probable, however, that two different oncogenic agents could be recovered from AK leukæmic extracts; one causing leukæmia in C3H mice, and another causing salivary gland carcinomas. Since salivary gland tumours have never been observed in mice of the AK line, whereas spontaneous leukæmia is

of the 35 inoculated mice, nine developed salivary gland carcinomas at an average age of 4·3 months, and two developed typical "spontaneous" leukæmia at 10·7 months of age (Table VI). It was evident from these experiments that

Table VI

RESULTS OF INOCULATION OF CENTRIFUGED (9,500 RPM) EXTRACTS PREPARED FROM NORMAL AK EMBRYOS, INTO NEWBORN C3H OR $C3H_f$ MICE

At age at inoc. (hr.)	*No. of Mice inoc.*	*No. of Mice Devel. Leuk.*	*Average age when Leuk. Devel. (months)*	*No. of Mice Devel. Sal. G. C.A.*	*Average age when Sal. G. C.A. Devel. (months)*
12	35	2	10·7	9	4·3

the leukæmic agent, as well as the tumour agent, is present in extracts prepared from normal AK embryos. These results suggest that both the leukæmic agent and also the agent causing salivary gland carcinomas are transmitted in mice of the AK line from one generation to another through the embryos.

Transmission of the AK Leukæmic Agent from One Generation to Another in Mice of the C3H Line

Following inoculation of newborn C3H mice with the AK leukæmic agent, *not only did most of the inoculated mice eventually develop leukæmia, but the agent apparently* passed to their offspring, causing also, at least in some of them, the development of "spontaneous" leukæmia. An attempt to originate experimentally a "leukæmic C3H line" has not succeeded, however. While almost 50 per cent of the untreated F_1 offspring of the inoculated C3H mice developed "spontaneous" leukæmia (Gross, 1951*b*), *the incidence of leukæmia in the second (F_2) and third (F_3) generations of offspring has been thus far very low*, leukæmia developing in some of these animals, if it developed at all, very late (Fig. 6), at twenty-two or twenty-three months of age (Gross, 1954). It is apparent that the C3H strain of mice may not be sufficiently

attempts to prevent the development of spontaneous leukæmia by foster nursing have also failed (MacDowell and Richter, 1935; Furth, Cole and Boon, 1942). Since it was evident, therefore, that the leukæmic agent is not transmitted through the milk, it was possible to assume that it *may be transmitted from one generation to another directly* through the embryos, if such a transmission occurs at all. In order to determine whether such an assumption is justified, normal embryos were removed aseptically from the wombs of young, healthy pregnant AK females; these embryos were then separated from the placenta, cut in small pieces with sterile scissors, and ground with physiological saline solution. The cell suspensions thus obtained were then used for inoculation, in one series of experiments. In another series, the normal AK embryo cell suspensions were centrifuged at 3,000 r.p.m. (fifteen minutes), then at 9,500 r.p.m. (ten minutes), and the final supernatant was used for inoculation. In 13 experiments a total of 57 newborn C3H$_f$ or C3H mice was inoculated, at an average age of thirteen hours, with the AK embryo cell suspensions. As a result, nine mice developed typical "spontaneous" leukæmia at an average age of thirteen months (16 per cent), and one additional mouse in this group developed a bilateral salivary gland carcinoma at four months of age (Table V). In another series of six experiments, the

Table V

RESULTS OF INOCULATION OF CELL SUSPENSIONS, PREPARED FROM NORMAL AK EMBRYOS, INTO NEWBORN C3H OR C3H$_f$ MICE

Av. age at inoc. (hr.)	*No. of Mice inoc.*	*No. of Mice Devel. Leuk.*	*Average age when Leuk. Devel. (months)*	*No. of Mice Devel. Sal. G. CA*	*Average age when Sal. G. CA Devel. (months)*
13	57	9	13	1	4

supernatant resulting from centrifugation (9,500 r.p.m.) of the AK embryo cell suspensions was inoculated into 85 newborn (less than twelve hours old) C3H$_f$ or C3H mice;

susceptible to the AK leukæmic agent: even though, following injection of newborn C3H mice with the AK leukæmic extracts most of the inoculated mice may develop leukæmia (Gross, 1951b and 1952), in successive generations of offspring of the inoculated mice the agent may become rather adapted to this particular strain, causing the development of leukæmia in subsequent generations only occasionally and at advanced age. Eventually, after several generations, it may either completely disappear, or it may become so submerged and masked as to lose, with only occasional exceptions, its ability to become activated spontaneously.

Is Leukæmia a "Communicable" Disease? A Working Hypothesis

The following working hypothesis is advanced to explain the experimental results thus far obtained —

It is possible to assume that in mice of the AK line, "spontaneous" leukæmia is caused by a cell-free agent, transmitted from one generation to another. Unlike the mammary carcinoma agent, however, the mouse leukæmia agent would be transmitted not through the milk of nursing mothers, but through the embryos, i.e. most probably directly through the fertilized ova. Mice developing from such infected ova would remain in good health through their early adult age, because the leukæmic agent, carried by such mice, would exist in an inactive form, frugal and moderate in its requirements, and therefore harmless for its carrier-hosts. Occasionally, *however, and particularly in middle-aged carrier-hosts*, the leukæmic agent, prompted by as yet obscure factors, could become activated. The activated agent would be highly pathogenic, causing rapid multiplication of cells harbouring it, and causing the development of leukæmia. The fate of the carrier host would thus be sealed. The survival of the leukæmic agent, however, would be assured, its transmission *to the host's offspring occurring prior to its activation in the* parent host.

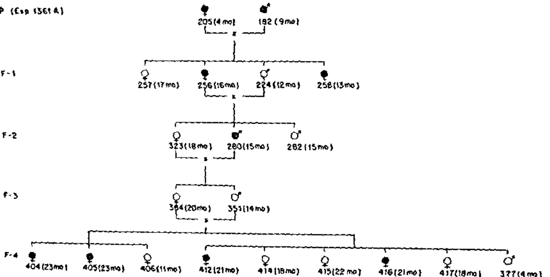


FIG 6 "Vertical" (from one generation to another) transmission of leukæmia in the C3H strain of mice. Only female 205 and her brother 182 had been injected (within a few hours after birth) with AK leukæmic extracts Their offspring were un-treated Black colour indicates the development of leukæmia. It is evident that leukæmia developed in several descendants of successive generations. (This strain of mice is essentially free from spontaneous leukæmia. The inoculation of the parent-couple 205 and 182 with the leukæmic agent apparently initiated a "vertical" transmission of this disease in this particular family of mice)

other malignant tumours developing in other members of the same family-tree. This working hypothesis may offer an explanation of clinical observations suggesting that in families of patients suffering from leukæmia, cancer is more common than in the average population. It would also offer an explanation for the occurrence of leukæmia or other malignant tumours in more than one member of the same family in human beings. Finally, should this hypothesis be correct, it would then have to be assumed that the number of individuals with symptoms of tumours or leukæmia would represent only a fraction of those actually carrying the seeds of either disease.

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DISCUSSION

FURTH Several of us have tried to demonstrate the virus of mouse leukæmia without success*. Now we ask ourselves just what is Dr Gross's trick The answer is the use of newborn baby mice for inoculation It was discovered in the course of studies of the milk tumour agent that newborn mice are the only susceptible hosts; using mice less

* For references to original publications, see the review article in *Physiol Rev*, (1946) 26, 47

Recent experiments suggest that mice of the AK line may carry at least two different oncogenic agents: one causing leukæmia, the other causing salivary gland carcinomas. Both agents are transmitted from one generation to another through the embryos, i.e. most probably directly through the germinal cells. Since salivary gland tumours never develop in AK mice, whereas spontaneous leukæmia is very common in these animals, it would follow that an interference phenomenon is responsible for the latency of the salivary gland tumour agent in the AK mice, or that the AK mice are not susceptible to the pathogenic action of this agent.

Some Possible Implications for Human Pathology

The theoretical implications of these observations may be of considerable importance for human pathology. It is possible to assume that in humans, as in mice and chickens, leukæmia may be caused by a cell-free agent, transmitted from one generation to another. In certain instances, at least, such an agent may in human beings also be accompanied by another, transmissible oncogenic agent. An interference phenomenon may be responsible for the latency of either, or both, in most of the carrier hosts. Moreover, these agents would exist usually in their inactive forms, harmless for their carrier-hosts. Occasionally, however, prompted by as yet obscure factors, possibly by some trigger mechanisms, not necessarily specific, an activation of either the leukæmic or the tumour agent may occur. It is quite conceivable that such an activation, being sometimes related to the hormonal balance, would more frequently occur either prior to adolescence, or after middle age, of the carrier host. The oncogenic agents, hitherto masked and harmless, would then change into formidable pathogens, causing rapid multiplication of cells harbouring them. This would result in the development of either leukæmia, or of another malignant tumour, depending on the individuality of the activated agent. In families carrying two or more oncogenic agents, one of them may cause leukæmia, while others may cause the development of carcinomas or

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Table II (FURTH)
TRANSPLANTATION TESTS

Donor leukæmic cells	Per cent "takes" in AK	Per cent "takes" in C3H
AK5	100	60–64
AK106	100	82–100
F_1: C3H/AK117	100	71–89
F_1. AK/C34 201	96	92
AK	0 per cent in C57 Black and RF	

Table II shows the closeness of the C3H and AK strains. Of all strains tested, C3H was the only one which took AK cells It is possible that the strains have changed since this work was done, and C3H is now more remote from AK, since Dr Gross could transplant AK cells only to newborn C3H [illegible]

generation hybrids had a low incidence of leukæmia, slightly more when the female partner was of the leukæmic strain. This would mean, in Dr. Gross's terms, that the virus had been transmitted also by the AK male. The F_1 and F_2 hybrids were crossed back to the AK's and RF's, and so on. The leukæmia incidence was greater when the AK female [illegible]

cording to Gross, only C3H mice, the closest to AK, take the AK virus If interpreted in Dr. Gross's terms, it would seem that what is inherited is resistance or susceptibility to this unique virus.

There is, however, another possibility Several investigators have described filtrable agents which can transfer (transduce) hereditary traits from [illegible]

chromosomes can float free and enter new host cells and create a new cell type. It is conceivable, fantastic as it appears to be, that leukæmic cells yield such free chromosomal determinants capable of entering

than ten hours old seems essential for leukæmia transfer, according to Dr. Gross. Another matter of possible importance is that he chills his material as well as his tools and does the filtration and centrifugation in the cold and carries out all procedures, including inoculation, with great rapidity. Taking the data which he presented as facts, I cannot avoid the conclusion that he has a filtrable agent producing leukæmia and an agent producing salivary gland tumour.

How are our earlier findings to be interpreted in the light of what Dr. Gross has shown? I have brought along three relevant lantern slides, now over ten years old.

Table I (FURTH)

PRELIMINARY TABULATION OF DATA ON THE MILK FACTOR OF BITTNER

Mice			*Incidence of Neoplasms, Per Cent*		
Stock		Number	Breast	Lung	Leukæmia
C3H	♀, Virgin	74	18		
	♀, Bred	114	55		
	♀ and ♂, Total	311		1.5	0.3
	Fostered by AK				
	♀, Virgin	63	7		
	♀, Bred	4	25		
	♀, and ♂, Total	148		0.5	0
AK	♀, and ♂, Total	198	0	0	74
	Fostered by H				
	♀, Virgin	69	0		
	♀, Bred	19	21		
	♀, and ♂, Total	163		0	34
AK♀ × C3H♂	♀, Virgin	92	1	1	57
	♂	95	0	0	61
C3H♀ × AK♂	♀, Virgin	93	10	4	44
	♂	82	0	2	33

Table I indicates that C3H mice very seldom have leukæmia (only 1 in 300 here), not even when fostered with AK mice (none in about 148). On the other hand, the AK mice fostered reciprocally developed breast carcinoma and had a reduced leukæmia incidence, but the latter dropped also in AK mice nursed by different AK mothers. The incidence of spontaneous leukæmia in the AK strain is very high. It is somewhat less in the first generation hybrids AK × C3H but is still very high. Since the AK male also transmits the disease it appears that the sperm contains the "virus", passing it on to the first generation hybrid.

leukæmia a virus, or have you?

GROSS: I can only state that leukæmia in mice (or at least in certain strains of mice, and under certain experimental conditions) is transmissible through a filtrable, thermolabile agent, which apparently multiples, this agent produces specific symptoms of disease, and, apparently it may also interfere with another oncogenic agent (salivary gland carcinoma. On this basis I assume, tentatively, that it behaves like certain other viruses.

Now, is the leukæmic agent "communicable"? We are conditioned to think of a disease being "communicable" only if it spreads very

worm, is transmitted through the ovum. We know now that many other virus diseases in plants and insects are transmitted in such a manner There are probably numerous infectious diseases, not necessarily in

leukæmic agent is, or at least was at one time, extraneous. Once acquired, it might have been carried, from one generation to another, within the host's cells, for many years, perhaps centuries

HADDOW To what extent have you tested strains other than C3H by the same technique?

GROSS: To a rather limited extent I injected some 60 mice of the C57Black line when less than twelve hours of age with the centrifuged AK leukæmic extracts As a result, some 35 per cent of the injected mice developed leukæmia, but very late, at sixteen to twenty-three months of age In addition, I also injected mice of the C57Brown line with AK leukæmic extracts

FURTH I don't know the C57Brown strain, C57Black mice rarely have leukæmia when young but may develop it at an advanced age.

GROSS: I have not seen spontaneous leukæmia in untreated C57-Brown mice When, however, centrifuged ($7000 \times g$) AK leukæmic

Table III (FURTH)

INCIDENCE OF LEUKÆMIA IN HIGH (AK) AND LOW LEUKÆMIA STRAINS (RF) AND HYBRIDS

A/A 69 3% A/R 21 9%	A/AR	24 8%[a]	AxAR/A	46 7%[c]
			AxAR/R	2 8%[d]
	AR/A	28 8%[a]	ARxA/A	50 0%[d]
			ARxA/R	7 0%[d]
	R/AR	2 6%[d]	RxAR/A	4 7%[a]
			RxAR/R	3 7%[a]
	AR/R	2 8%[d]	ARxR/A	8 6%[a]
			ARxR/R	1·7%[a]
	AR/AR	11 8%	ARxAR/ARxAR	7·5%
			ARxAR/A	14 5%[b]
			ARxAR/R	4 1%[d]
R/R 1 6% R/A 11 6%	RA/RA	8 6%	RAxRA/A	13 4%[a]
			RAxRA/R	5·7%[a]
			RAxRA/RAxRA	6 3%
	RA/A	13 5%[a]	RAxA/A	32 2%[d]
			RAxA/R	4·3%[c]
	A/RA	27 0%[d]	AxRA/A	38·3%[a]
			AxRA/R	10 1%[d]
	RA/R	4 2%[b]	RAxR/A	11 2%[b]
			RAxR/R	0 0%[b]
	R/RA	1 8%[d]	RxRA/A	10 1%[d]
			RxRA/R	8·9%[a]

The female is always on the left of each pair or sign For backcross generations: a = no significant change, b = significant change. $P = < 0\ 05$; c = significant change $P = < 0\ 02$, d = highly significant change $P = < 0\ 01$

into the heredity of lymphoid cells of the newborn. Thus, Dr. Gross, it is conceivable that the genetic theory can be reconciled with the virus theory.

GROSS May I add the following experiments to the data which I presented: normal testicles were removed from young (six to eight week-old) healthy AK males, ground with physiological saline solution, and injected into newborn C3H mice As a result, several of the inoculated animals later developed leukæmia This observation was consistent with the assumption that the AK leukæmic agent may be present in AK sperm Further experiments are still needed, however, to determine this point.

leukæmia a virus, or have you?

Furth No, I won't quibble about words, once I antagonised a senior friend, Dr. Murphy, by calling the filtrable leukæmia-producing agent of chickens a virus, thereby siding with Dr. Rous, who then believed that these agents possessed all attributes of common viruses. Call it what you wish

Gross· I can only state that leukæmia in mice (or at least in certain strains of mice, and under certain experimental conditions) is transmissible through a filtrable, thermolabile agent, which apparently multiples, this agent produces specific symptoms of disease, and, apparently it may also interfere with another oncogenic agent (salivary

worm, is transmitted through the ovum We know now that many other virus diseases in plants and insects are transmitted in such a manner There are probably numerous infectious diseases, not necessarily in

though this is merely a speculation, that the mouse leukæmia agent attaches itself to the chromosome. Would that be a genetic factor then? Not necessarily so I would rather be inclined to assume that the leukæmic agent is, or at least was at one time, extraneous Once acquired, it might have been carried, from one generation to another, within the host's cells, for many years, perhaps centuries

Haddow To what extent have you tested strains other than C3H by the same technique?

Gross To a rather limited extent I injected some 60 mice of the C57Black line when less than twelve hours of age with the centrifuged AK leukæmic extracts As a result, some 35 per cent of the injected mice developed leukæmia, but very late, at sixteen to twenty-three months of age In addition, I also injected mice of the C57Brown line with AK leukæmic extracts

Furth I don't know the C57Brown strain, C57Black mice rarely have leukæmia when young but may develop it at an advanced age.

Gross I have not seen spontaneous leukæmia in untreated C57-Brown mice. When, however, centrifuged ($7000 \times g$) AK leukæmic

extracts were inoculated into newborn (less than twelve hours old) C57Brown mice, some 25 per cent of the injected animals developed typical leukæmia at eight to fifteen months of age. This C57Brown leukæmia was then transplantable, by cell implantation, to C57Brown

leukæmic give rise to a second and third leukæmic generation, and that this leukæmia is not transplantable to AK mice, then I became convinced that he has an agent capable of inducing leukæmia

HADDOW: Yes, I think I should feel the same way. And also there is the transmission, as I gather, from normal embryonic extracts

Apart from the question of the nature of the leukæmia-producing agent, you have shown in your first experiment the transmission of leukæmic cells from AK to C3H, and the sensitivity to such of the infants and not of the parents. That fits in with what has already been known for a very long time as to the relative immunological undevelopment in infants of that age

GROSS: Adult C57Brown mice are for all practical purposes completely resistant to inoculation of AK leukæmic cells. On the other hand, newborn C57Brown mice are perfectly susceptible. Following implantation of AK-leukæmic cells into newborn C57Brown mice, leukæmia develops within two or three weeks. This is, however, only a graft, since such a leukæmia is transplantable readily back to the donor AK strain, but not to C57Brown adult mice. In order to produce a true C57Brown leukæmia (transplantable to C57Brown mice), centrifuged AK leukæmic extracts must be inoculated into newborn C57Brown mice. The leukæmia which will then develop a year or so later in such mice will consist of C57Brown cells, i.e. it will be readily transplantable by cell graft to adult C57Brown mice, but not any more back to the donor AK strain.

HADDOW: I wondered if I might suggest that before Dr. Gross goes back he could acquaint himself with Medawar and Billingham's recent

new type of experiment challenging these pre-treated mice with the agent at a later stage, say at four months

FAGRAEUS: We might consider analogies from other virus work.

cerebrally failed to change the properties of the virus, since material

strains, such as the C58, which has 80-90 per cent spontaneous leukæmia?

GROSS: I have a recent series of experiments with C58 donors, still in progress. There are no results yet, except perhaps one, still questionable*.

symptoms.

GORER: No symptoms, no. But the woman got no symptoms either. I know analogies are never exact, but she may have developed a viræmia transiently early in the pregnancy and infected the fœtus.

FURTH: Not if the virus can be carried with the sperm? It has not been shown that a C3H female once mated with AK sperm would, on

* ADDENDUM

leukæmia.

in which the pregnant female was injected at the time of pregnancy with this extract? Then she would get the viræmia, but presumably it shouldn't carry on to the offspring.

Gorer Another point in which I was interested was the antigenic similarity between the AK and the C3H. Isn't it possible that these antigens, which we know little about, are in fact virus receptors, and are what the virus attaches itself to, and that is why you can transmit from AK to C3H? If you have a highly specific virus which will go on to some receptors and not others, you would get genetic segregation.

Law: In regard to Dr. Gorer's suggestion, there is known to appear in certain low leukæmic strains of mice what is known as the "maternal

resistance factor That may be an important point.

But I think we ought to keep in mind that there is a balance in ætiological factors. I would draw your attention to the situation in breast cancer in mice At the time it was shown there was a milk agent, all the emphasis was placed on this virus-like agent, but now we know that there are genetic factors which relate to susceptibility of the mammary tissues to the virus, transmission of the agent and hormonal stimulation in its various aspects It is possible to inject with the viral agent without production of mammary tumours. I think it is important to determine whether, and how much, leukæmia in mice is influenced by this virus-like agent, and what part it plays in the ætiology of leukæmia There can be no doubt from the experiments of MacDowell and Furth and Cole that genetic factors are very important, and are probably related to susceptibility.

I should like to add that we have done quite a lot of work along the lines of Dr Gross's experiments We followed his techniques and we have also used other techniques We do get these anaplastic parotid tumours using AK or C58 leukæmic tissue in C3H mice, but I don't believe you have to follow the precise techniques which Dr Gross specifies. We find parotid tumours, using cell homogenates, centrifugates, or filtrates. These tumours are like those he described, very pleomorphic with many spindle cells and suggestion of glandular reproduction I hope we are able to find some leukæmias among the test mice soon.

Furth How long have your animals gone after injection of filtrate?

Law. We have some animals that are two years old or more. Of course there are a lot that aren't more than eight months or less.

Engelbreth-Holm In how many animals did these parotid tumours develop?

not always bilateral They resembled very much the type of tumour of the parotid occasionally seen developing spontaneously in myoepithelioma. If they arise early, they resemble very much the description of [illegible]

leukæmic cell suspensions. For instance, cell suspensions prepared from normal AK spleens produced in some instances salivary gland tumours, following inoculation into newborn C3H mice AK leukæmic cell suspensions heated to 63°C also occasionally produced salivary gland carcinomas in C3H mice. However, on the basis of experiments thus [illegible]

C3H mice.

[illegible]

twenty three months only. In order to obtain a more prompt develop- [illegible]

[illegible]

you yesterday, by the fact that if we treated our strain of mice with benzene, we only got lymphosarcoma in some animals. But if you have this leukæmia agent in these strains you might get it in a much higher percentage.

[illegible]

be true in the case of AK mice, we have a stage of [illegible]

i e pathogenic, unless prompted by such inducing triggers as X-rays or other carcinogenic factors

in which the pregnant female was injected at the time of pregnancy with this extract? Then she would get the viræmia, but presumably it shouldn't carry on to the offspring.

GORER Another point in which I was interested was the antigenic similarity between the AK and the C3H. Isn't it possible that these antigens, which we know little about, are in fact virus receptors, and are what the virus attaches itself to, and that is why you can transmit from AK to C3H? If you have a highly specific virus which will go on to some receptors and not others, you would get genetic segregation.

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mammary tissues to the [illegible]
stimulation in its various [illegible]
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FURTH. How long have your animals gone after injection of filtrate?

LAW· We have some animals that are two years old or more. Of course there are a lot that aren't more than eight months or less.

ENGELBRETH-HOLM *In how many animals did these parotid tumours develop?*

[illegible]

litter in which all the members developed parotid tumours) they were

THE PHENOMENA OF RESISTANCE AND DEPENDENCE IN LEUKÆMIC CELLS

L. W. LAW

It is pertinent and encouraging to note that the compounds which are carcinostatic to leukæmias of the mouse have been shown to be carcinostatic in man and *vice versa*. Two groups of compounds, classed as anti-metabolites, have been used to a great extent in the treatment of acute childhood leukæmias and in laboratory investigations employing this morphological form of leukæmia in mice: (1) folic acid antagonists, particularly those with a 4-amino substituent, and (2) purine antagonists. Folic acid antagonists have been found in our laboratory to be anti-leukæmic agents to every lymphocytic leukæmia tested On the other hand, purine antagonists are effective anti-leukæmic agents in some, but not other, leukæmias.

Folic analogues are of interest for the following reasons· (1) They are the most efficacious of known anti-leukæmic agents, (2) selectivity of action is in certain cases striking, and (3) the inhibitory effect can be shown to be of a competitive nature, affording a better means of elucidating the metabolic reactions involved in therapeutic investigations

The failure, after a period of time, to achieve remissions in patients with the compounds mentioned is a common observation. In experimental leukæmia it is shown that these failures result from the development of transformations in the population of leukæmic cells to resistance and (or) dependence.

It is the purpose of this report to consider the experimental production of these transformations, some characteristics of the transformed leukæmic cells, investigation of the manner of origin of these variant cells and some basic information that has been obtained involving the mechanisms of these phenomena.

FURTH: Every strain develops leukæmia after exposure to ionising irradiation, so we must suppose that every strain carries your virus.

GROSS: It would not necessarily be impossible to assume that many, perhaps most, strains carry latent oncogenic agents. We could perhaps compare latent oncogenic agents with other latent viruses, such as the bacteriophage. I wonder whether there are many strains of microbes that do not carry at least one bacteriophage, or a prophage. Could we not assume that latent oncogenic agents are equally common in nature?

BURCHENAL: Hasn't Kirschbaum shown that different stocks of mice develop different incidences of leukæmia after X-ray? For instance it is my impression that C3H has a much lower incidence after irradiation than some of the other strains.

LORENZ: I don't think that is quite correct. It depends on the method of treatment. We find that a single dose in C3Hf mice even at birth gives rise to only a few leukæmias, but if chronic irradiation of 8·8 r eight hours per day is given throughout life one gets 33 per cent leukæmias. You have to have the proper irradiation conditions for each strain to induce leukæmia.

FURTH: True, but there are also strain differences.

dependence. Table II shows the behaviour of these transformed cells in comparison with the sensitive control line, using the criterion of localized growth of lymphomatous

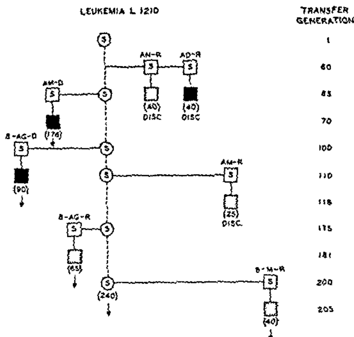


FIG 1 Showing origin of various transformed sublines discussed in text· AN-R (resistant to Aminopterin), AD-R (dependent on Adenopterin) AM-D (dependent on Amethopterin), 8-AG-D (dependent on 8-azaguanine), AM-R (resistant to Amethopterin), 8-AG-R (resistant to 8-azaguanine), 6-M-R (resistant to 6-mercaptopurine) Hatched squares represent resistance, darkened squares, dependence The control line, represented by circles, has been carried through 240 consecutive transfers and remains sensitive to the antifolic and antipurine compounds Certain transfer lines have been discontinued as shown, others have been carried serially through the number of transfers designated below the square

tissue, and Table III the behaviour in the development of generalized leukæmia following intraperitoneal transfer of a standard dose of leukæmic cells in Locke's solution (8×10^6 cells). It is of interest to note that even at a dosage level of

Folic Acid Analogues

Both types of transformation—to resistance, where leukæmic cells grow optimally *in vivo* either in the presence or absence of antagonist, or to dependence, where the cells grow optimally only in the presence of antagonist—have been obtained using the 4-amino-substituted folic analogues, 4-amino PGA (Aminopterin), 4-amino-N^{10}-methyl PGA (Amethopterin), 4-amino-9-methyl PGA (Aminopterin), and 4-amino-9, N^{10}-methyl PGA (Adenopterin) (Law, 1951*a* and *b*; Law and Boyle, 1950).* (See Fig. 1 and Table I.)

Variant sublines of transplantable acute lymphocytic leukæmias are obtained with ease following consecutive serial transfers in mice receiving either (1) near maximum tolerable levels of antagonist or (2) periodic increases in the level of antagonist. Once transformation has been achieved the variant lines retain their characteristics; no reversion to sensitivity has been observed or changes from one type of transformation to another. These characteristics are maintained in the absence of the antagonist used to achieve the transformation. The character is thus shown to be stable, irreversible and heritable

One particular subline of an acute lymphocytic leukæmia, L1210 in DBA/2 strain mice, has been of interest in determining some of the physiological characteristics of these transformed cells, the AM-D subline, dependent on folic antagonists for optimal growth

Characteristics of Leukæmic Cells Dependent on Folic Analogues for Optimal Growth

The AM-D variant line has now been carried through more than 175 consecutive serial transfers in DBA/2 strain mice. Optimal growth is obtained in the presence of 4-amino-N^{10}-methyl PGA (Amethopterin), the analogue used to develop

*Folic acid analogues and 8-azaguanine kindly supplied through the courtesy of Lederle Labs Division, American Cyanamid Co, antipurines through the courtesy of Dr George Hitchings, Wellcome Research Lab.

Table III

COMPARISON OF RESPONSE TO AMETHOPTERIN OF DEPENDENT (AM-D) AND SENSITIVE LEUKÆMIC CELLS* OF LEUKÆMIA L1210

Transfer line	Dosage Daily	(mg/kg) Total	Mean tumour wt mg	Peripheral blood counts: Hb	wbc	agran	gran	blasts	Organ infiltrations: Lymph nodes	spleen	liver
Dependent	3	12	1,362 7	10 1	47,760	11,040	32,209	2,821	+ + +	+ + +	+ +
	5	20	1,146	12	24,930	7,719	16,000	1,211	+ + +	+ + +	+ +
	7 5	30	1,084	12	46,835	9,835	34,190	2,810	+ +	+ +	+ +
	12	48	685 7	13 2	6,685	1,872	4,783	30	+ +	+	——
Sensitive	0	0	1,478	11 2	41,150	14,568	20,821	5,761	+ + +	+ + +	+ +
	3	12	0	13 5	11,025	6,550	4,520	55	——	——	——

*Transfer generation 100 of sensitive cells and comparable generation of dependent cells used

+ + + Severe, + + moderate, + slight, —— no infiltration, of leukæmic cells into designated organs as determined by microscopic study.

Amethopterin as high as 12 mg./kg. (total dose, 48 mg./kg.) an inhibition of localized growth of lymphomatous tissue of, 50 per cent occurs, but infiltration into lymph nodes and

Table I

TRANSFORMATIONS IN LEUKÆMIC CELLS OF SEVERAL TRANSPLANTABLE LINES

Line of Leukæmia	*Strain of Mouse*	*Type of Transformation*		*Antagonist used*
		Resistant	*Dependent*	
L1210	DBA	+	+	Amethopterin
L1210	DBA	+	—	Amethopterin
L1210	DBA	+	+	Adenopterin
L1210	DBA	+	—	Aminopterin
L3054	C58	+	—	Adenopterin
HE8186	A	+	?	Amethopterin
L4946	AKR	+	—	Amethopterin
L1210	DBA	+	+	8-azaguanine
L1210	DBA	+	—	8-azaguanine
L1210	DBA	+	—	6-mercaptopurine

Table II

DEPENDENCE IN LEUKÆMIC CELLS OF THE AM-D SUBLINE OF LEUKÆMIA L1210

	No. Mice	*Amethopterin Dosage (mg./kg.)* *Indiv.*	*Total*	*Mean wt. Lymphomatous Tissue mg.*
Dependent	20	12	48	685 5
	78	3	12	833 3
	29	None		212 7
Sensitive	20	12	48	0
	30	3	12	6 0
	55	None		1,190 0

Transfer generations 98–108 of sensitive cells and 33–39 of dependent cells used.

spleen was evident. This indicates at least a fifty-fold increase in tolerance to this antagonist, since it requires 0 25 mg./kg. (total dose 1·0 mg./kg.) of antagonist to inhibit localized growth of the sensitive cells to a similar degree.

Amethopterin on sensitive leukæmic cells and (2) the optimal growth-promoting capacity of this antagonist on dependent leukæmic cells. It may be seen by reference to Table V that partial reversals of both the anti-leukæmic effect in the sensitive line and of the growth promoting effect on the dependent line were obtained. Folic acid (PGA) was also

Table V

CITROVORUM FACTOR (CF) AND THE EFFECTS OF AMETHOPTERIN ON SENSITIVE AND DEPENDENT (AM-D) LEUKÆMIC CELLS OF LEUKÆMIA L1210

Experiment	*No Mice*	*Dosage**		*Relative mean† weights*
		Daily	*Total*	
		Dependent (AM-D)		
Amethopterin	42	2	8	1
Amethopterin + CF	28	2 2×10^6	8 10×10^6	0 71
CF	36	2×10^6	8×10^6	0 35
Controls	36	0	0	0 31
		Sensitive		
Amethopterin	54	2	8	0 03
Amethopterin + CF	44	2 2×10^6	8 10×10^6	0 28
CF .	30	2×10^6	8×10^6	0 91
Controls .	50	0	0	1

*Dosage of Amethopterin in mg /kg citrovorum factor dosage in units/kg
†For convenience, optimal growth in both groups considered as 1 0

found to give similar reversals. The ratio of analogue to metabolite (PGA) for maximum effect was approximately 1 . 15 and the ratio was the same for sensitive or dependent cells providing that PGA was always given prior to administration of analogue PGA and CF were found not to influence the growth characteristics of either sensitive or dependent cells, under the conditions of these experiments

No changes in morphology, antigenicity or transplantability have been observed in the several variant forms of resistant or dependent cells developed in leukæmia L1210 or the other transplantable leukæmias employed Leukæmic cells of the

A specific cross-dependence is shown in Table IV. Any 4-amino substituted folic antagonist is capable of providing for optimal growth. Some of the so-called "weak" antagonists, notably N^{10} methyl PGA and 9-methyl PGA, though lacking anti-leukæmic activity, are also able to provide for approximately 50 per cent optimal growth of these dependent cells. Such anti-leukæmic compounds as 8-azaguanine, α-peltatin,

Table IV

COMPARATIVE SENSITIVITY OF AMETHOPTERIN-DEPENDENT (AM-D) AND SENSITIVE LEUKÆMIC CELLS TO SEVERAL ANTI-LEUKÆMIC AGENTS

Compound	*Dosage (mg /kg)*		*Sensitive*	*Dependent*
	Indiv.	*Total*	*Inhibition index**	
8-azaguanine	50	400	0 13	0 17
α-Peltatin	5 0	15	0 06	0 06
TEM	0 75	3 0	0 27	0 08
6-Mercaptopurine	75	525	0 10	0 10
Aminopterin	3	12	0 04	0 94
Adenopterin	3	12	0 01	1·03
Amino-an-fol	30	120	0 20	0 78
Aminopterin	0 2	0 8	0 01	1 0

*Inhibition index = $\frac{\text{Mean wt tumour treated}}{\text{Mean wt tumour controls}}$

TEM and 6-mercaptopurine, on the other hand, show independence of action, in being carcinostatic for either the dependent or sensitive sublines Two compounds, cortisone and a purine antagonist, 2, 6-diaminopurine, were ineffective in either the dependent or sensitive subline in this study.

The 4-amino-substituted folic analogues appear to inhibit leukæmic-cell growth by antagonism of folic acid (PGA) or citrovorum factor (CF) since either of these compounds will prevent anti-leukæmic action of this class of agents (Burchenal *et al.*, 1950). Since it appears that CF, on a weight basis, is more effective than PGA in reversing the effects of folic analogues, it was of interest to study the effects of this metabolite on the growth characteristics of the sensitive and dependent lines of leukæmia L1210 and to determine the blocking effect of CF on (1) the anti-leukæmic action of

utilization of these analogues by *S. fæcalis* and *Tetrahymena* and that the explanations given above are not valid.

In considering explanations for the dependence characteristic, it is apparent that many of the suggestions pertaining to resistance are unlikely. For example, the ability of leukæmic cells to detoxify the antagonist or to convert antagonist to PGA or CF would explain the phenomenon of resistance but not dependence. Preliminary trials by Nichol (1953) on the ability of our L1210 Amethopterin-dependent (AM-D) leukæmic cells to convert PGA to CF indicate that the dependent leukæmic cells are less active than sensitive cells in this respect, in contrast to the results obtained with *S. fæcalis*. It is quite likely that differential absorption of folic analogue or PGA is not related to the dependence phenomenon Preliminary trials by Skipper and colleagues (Skipper, 1953) using ^{14}C-labelled PGA and Amethopterin show ^{14}C contents of sensitive and dependent L1210 leukæmic cells to be of the same order.

It is possible that the dependent leukæmic cells described here have acquired the ability to use folic analogues without conversion to PGA or CF, employing a different mechanism for the synthesis of nucleic acids than that suggested as occurring normally. Certain preliminary data are available relating to this interpretation As mentioned previously, it is evident that the 4-aminopteroylglutamic acids act by competing with PGA or CF It appears that PGA is converted to CF, which is associated with enzymes concerned with transfer of single carbon units Folic antagonists compete with formed CF, and the end result of this antagonism is an inhibition of nucleic acid synthesis as well as other biochemical processes. It has been found (Skipper, Bennett and Law, 1952), using Na Formate-^{14}C (a precursor of the 2- and 8-carbon atoms of nucleic acid purines) that the folic acid antagonist, Amethopterin, inhibits *de novo* synthesis of DNA and RNA purines in the sensitive cells and viscera of mice bearing these cells, while the analogue more than doubles the rate of *de novo* DNA and RNA synthesis in the dependent

sensitive and of the transformed sublines, as observed in localized growth or in infiltrations into spleen, liver, lymph nodes or in the peripheral blood are morphologically indistinguishable. Attempts to detect antigenic differences by complement-fixation tests have been unsuccessful.

Possible Mechanisms of Resistance and Dependence

Resistance of leukæmic cells to folic analogues may indeed be the result of (1) a lowered PGA requirement accompanied by a much greater capacity to convert PGA to utilizable CF, (2) an increased ability to detoxify the PGA antagonist, (3) a more efficient utilization of CF due to changes in permeability of the cell, or (4) the ability of transformed cells to convert antagonist to PGA or CF by one of several methods, deamination, demethylation, etc. In the case of *Streptococcus fæcalis*, resistant to folic antagonist, it appears that this strain has a lowered requirement for PGA and a much greater capacity to convert PGA to CF than the antagonist-sensitive strain, so that significant inhibitions of growth were obtained only with very high concentrations of antagonist (Hutchison and Burchenal, 1953; Nichol *et al.*, 1953). It is possible that the ability of the resistant cell to absorb analogue is greatly reduced

It has been reported recently (Hutchison and Burchenal, 1953, Broquist, 1952) that a folic-antagonist resistant strain of *S. fæcalis* was able to use Aminopterin and related analogues for growth, in contradistinction to the sensitive strain, by converting the analogues to PGA or CF. Similarly, Kidder *et al.* (1951) observed that the protozoon, *Tetrahymena*, was able to use Aminopterin (and Methopterin) in growth processes, suggesting that this organism also possessed enzymes capable of deaminating and demethylating these antagonists. It has been found by Nichol however, employing paper chromatographic techniques, that the folic acid antagonists which are presently available contain sufficient PGA and pteroic acid as impurities to account for the apparent

Table VI

INCORPORATION OF ^{14}C-FORMATE INTO NUCLEIC ACID MOIETIES OF VISCERA AND AMETHOPTERIN-SENSITIVE AND DEPENDENT (AM-D) TUMOUR MASSES OF LEUKÆMIA L1210

Group No	Leukæmia	Tissue	Treatment	Specific activities (μc./mole C)					
				Original tissue	DNA			RNA	
					Guanine	Adenine	Thymine	Guanine	Adenine
1	Sensitive	Viscera	None	6.0	171	280	—	199	248
	Sensitive	Tumour	None	4.2	206	227	100	309	308
2	Sensitive	Viscera	Amethopterin*	5.3	20	10	4	70	90
	Sensitive	Tumour	Amethopterin*	1.8	34	39	10	54	112
3	Dependent	Viscera	None	7.3	113	99	—	156	140
	Dependent	Tumour	None	1.8	55	51	24	85	86
4	Dependent	Viscera	Amethopterin†	4.9	15	10	3	37	35
	Dependent	Tumour	Amethopterin†	4.8	127	115	51	162	156

*Amethopterin (3 mg./kg.) immediately before $H^{14}COONa$ injection on seventh post inoculation day
†Amethopterin (3 mg./kg.) on 3rd, 5th, and 7th days. $H^{14}COONa$ on 7th day

leukæmic cells, profoundly inhibiting the nucleic acid synthesis in the viscera of mice bearing these transplanted cells. These results are shown in Table VI.

Similar results were obtained from *in vitro* studies of sensitive and dependent leukæmic cells from the same sources. Amethopterin at concentrations of 0·01 mg./ml. strongly inhibited the incorporation of ^{14}C formate into the protein and purine pentose nucleotides of sensitive cells. Amethopterin at much higher levels was found to be ineffective on the *in vitro* incorporation of $^{32}PO_4$. These results indicate that the effect of folic analogues is not an overall inhibition of tissue metabolism (Williams *et al.*, 1954).

It should be pointed out that, although the available folic antagonists contain certain contaminants which are growth factors, it is not necessarily a complicating factor in the production and development of transformations to resistance and dependence (Nichol *et al.*, 1953). On the contrary, it would appear that the presence of PGA in the antagonist used in *S. fæcalis* experiments aided in the selection of resistant mutants. It should be recognized, however, that some confusion in the interpretation of results has arisen.

Purine Analogues

Since the report of Kidder *et al.* in 1949 showing the cancerostatic effect of a triazolopyrimidine analogue of guanine, 8-azaguanine, on certain adenocarcinomas and a leukæmia in mice, this compound has been studied rather extensively. It has proved to be a useful and interesting tool in investigations of cellular biochemical reactions. Although specific and definite inhibitory action has been noted for a fairly wide range of morphological forms of neoplasm (Gellhorn *et al.*, 1950; Sugiura *et al.*, 1950; Law, 1950), it is equally clear that it is entirely inactive against other neoplasms in the mouse and rat (Gellhorn *et al.*, 1950, Sugiura *et al.*, 1950).

In certain acute lymphocytic leukæmias of the mouse (Law, 1950), particularly the transplantable leukæmia L1210, a definite, regular and reproducible inhibition of growth

sensitive subline die at 7·9±0·06 days, and when given 8-azaguanine parenterally at 12·4±0·20 days.

This dependent subline has now been carried through 90 transfer generations since emergence of the trait, and has retained its characteristic response without evidence of reversion

Cross-dependence on other purine analogues has been demonstrated in this 8-azaguanine-dependent line. 6-Mercaptopurine and thioguanine, both moderately carcinostatic, 8-azaguanine and 2 : 6-diaminopurine, ineffective for sensitive leukæmic cells, all provide for 50 per cent or more optimal growth of the dependent line. The folic antagonist, Amethopterin, and TEM are inhibitory for the dependent cells as well as the sensitive. A striking sensitivity to folic analogues of the dependent cells, as well as of other transformations produced by purine analogues has been noted and will be discussed later.

L1210 leukæmic cells transformed to resistance, using 8-azaguanine, grow optimally in DBA/2 mice with or without this antagonist, and the mice die from leukæmia at 10 1± 0 15 days Cross-resistance to all other purine analogues has been demonstrated (see Table VIII) but these resistant cells remain sensitive to other unrelated compounds such as folic analogues and TEM This line has now been carried through

Table VIII

INFLUENCE OF SEVERAL PURINE ANTAGONISTS ON VARIANT SUBLINES OF LEUKÆMIA L1210

Antagonist	*Sensitive Per cent*	*8 AG D Per cent*	*8 AG R*	*6 M-R*
8-azaguanine	+(100)	−(100)	0	0
6-Mercaptopurine	+(80)	−(60)	0	0
2 · 6-Diaminopurine	O	−(50)	0	0
8-azaxanthine	O	−(50)	0	0
Thioguanine	+(80)	−(40)	0	0

8-AG-D = 8-azaguanine-dependent; 8-AG-R = azaguanine-resistant, and 6M-R = 6-Mercaptopurine-resistent
+ = inhibition, − support of growth (cross-dependence)
O = no influence (cross-resistance).

results from parenteral administration of the guanine antagonist at dosage levels far below the maximum tolerable dose.

Transformations to resistance and to dependence have been obtained in leukæmic cells of line L1210 by consecutive serial passage in DBA/2 mice receiving near MTD levels of 8-azaguanine (see Fig. 1.) The dependent line (8AG-D) was developed from the 100th transfer of sensitive cells and a resistant line from the 175th transfer (Law, 1951c).

Transformations to Resistance and Dependence using 8-Azaguanine

Table VII shows the characteristics of dependence in the transformed cells. Optimal growth, as determined by

Table VII

Effect of 8-azaguanine on Sensitive and 8-azaguanine-dependent (8AG-D) Lines of Leukæmia L1210

Transfer line	*Number of mice*	*Dosage (mg /kg.)*		*Tumour wt at 9 days (mg.)*
		Daily	*Total*	
Dependent	24*	150	1,200	591 1
	183	75	600	533 6±41 1
	89	None		240 4±21 8
Sensitive	10	150	1,200	0
	53	75	600	16 8± 2 2
	54	None		775 6±80 2

*Transfer generations 107–144 of the sensitive line and 7–44 of the dependent line used

localized growth of lymphomatous tissue, was obtained at dosage levels of 8-azaguanine as high as 150 mg./kg. (total dose, 1200 mg /kg.). At this level complete inhibition of growth is observed in the sensitive line. Leukæmic death, following intraperitoneal transfer of cells, also strikingly reflects the dependence characteristic. The mean survival time of mice bearing the dependent subline was 15 8±0 45 days. If these mice are given 8-azaguanine (*75 mg /kg.×8*) they die earlier, 12 1±0 23 days with a florid leukæmia (see Table IX). In contrast DBA/2 mice bearing the control

been demonstrated by Heinrich *et al.* (1952) in the protozoon *Tetrahymena*, by Mitchell *et al.* (1950) for viscera of mice and more recently, using finer techniques, for mouse viscera and tumour tissue by Skipper (1953). The incorporation is for the most part in RNA and in relatively small amounts. There appears to be little doubt also in *Tetrahymena*, which requires preformed guanine, that the guanine antagonist acts strictly as an antimetabolite, physiological guanine or its nucleotide reversing in competitive fashion the growth-inhibiting capacity of 8-azaguanine. Evidence for a clear-cut metabolite-antimetabolite relationship in mice or other mammals is not yet available. Guanine has been shown to reverse the carcinostatic effect of 8-azaguanine, as determined by leukæmic deaths in mice (Law, 1950). Goldin *et al.* (1950) have shown that guanylic acid apparently is more effective than guanine, using the criterion of tumour volume, in leukæmia of mice. In our own observations with the 8-azaguanine-dependent leukæmia, guanylic acid regularly interferes with the growth-promoting capacity of 8-azaguanine (Law *et al.*, 1953) more effectively than another ribotide, adenylic acid It has not been determined if this is done competitively. On the other hand Gellhorn *et al.* (1954) have observed in rabbits bearing the Brown-Pearce carcinoma that the carcinostatic effects of 8-azaguanine are more easily reversed by the nucleosides and nucleotides of adenine, suggesting that 8-azaguanine is converted first to adenine prior to conversion to a riboside.

Extensive attempts in our laboratory (Law, 1953) to reverse the carcinostatic activity of 6-mercaptopurine by physiological purine bases have been relatively unsuccessful, although on occasion reversals have been obtained In *Lactobacillus casei* any of the four physiological purines will easily and competitively prevent the inhibition produced by this compound (Elion *et al*, 1951). The negative outcome of reversal experiments with 6-mercaptopurine does not necessarily mean that its mode of action is different in these experimental animals as compared with *Lactobacillus*, but that the techniques employed in the complicated system in experimental animals are not

65 consecutive serial passages in DBA/2 mice, retaining its characteristics.

Resistance to an Adenine Antagonist, 6-Mercaptopurine

The adenine analogue, 6-mercaptopurine, has been shown to act as a purine antagonist in the metabolism of *Lactobacillus casei* (Elion and Hitchings, 1953). It has also been shown to be a unique inhibitor of Sarcoma 180 (Clarke *et al.*, 1953) and of certain mammary adenocarcinomas (Skipper, 1953). Limited clinical trials of this compound in advanced leukæmia of children have been encouraging (Burchenal *et al.*, 1953). As with 8-azaguanine this compound has been shown to give definite, regular and reproducible inhibition of leukæmic cell growth in certain lymphocytic leukæmias but not others (Law, 1953). Striking increases in survival time in leukæmia L1210 occur. The mean survival time of mice bearing the sensitive subline of leukæmia L1210 was $7 \cdot 9 \pm 0 \cdot 06$ days, and an increase in survival of 87·3 per cent to $14 \cdot 8 \pm 0 \cdot 18$ days was obtained using this antagonist within the total dosage range of 250–1200 mg./kg. The effects obtained at the higher dosage levels (near MTD) were within the same range as those obtained at lower levels, 300–600 mg./kg.

A resistant line was procured starting with the 200th transfer of the sensitive line. The resistance characteristic was apparent after five consecutive transfers in DBA/2 mice receiving 75 mg./kg. × 7 dosage levels. No influence of 6-mercaptopurine could then be demonstrated in this line; test mice with and without antagonist dying at $9 \cdot 9 \pm 0 \cdot 10$ days. Cross-resistance, similar to that observed in the 8-azaguanine-resistant line, was found using other purine analogues (Table VIII) but sensitivity to TEM and Amethopterin was evident. A considerably increased sensitivity to the folic antagonist was characteristic.

Mode of Action of Purine Antagonists

There appears to be little doubt that the purine antagonist 8-azaguanine is incorporated into nucleic acids. This has

Origin of Resistance in Leukæmic Cells to Antimetabolites

It appears extremely likely that the transformations to resistance or dependence observed in leukæmic cells of the mouse occur spontaneously and rather generally among populations of leukæmic cells; the role of the antimetabolite is merely that of a selective agent (Law, 1952*a*). Increases in resistance have been shown to occur in a discrete step-wise fashion (Law, 1952*a*) resembling in this respect the development of resistance to penicillin in bacteria (Demerec, 1948). It is impossible to determine with these somatic cells whether the observed transformations are genetic. In *Escherichia coli*, strain K12, a sexually fertile strain, it has been shown that the numerous changes to resistance and dependence, using streptomycin, appear to have arisen by change at a single gene locus (Newcombe and Nyholm, 1950). Thus, these traits in bacteria behave as if controlled by allelic forms of the same gene locus.

Practical Considerations

Out of these studies have come two avenues of approach to the chemotherapy of leukæmia which appear to be of utmost significance. The first relates to changes in sensitivity to folic analogues of leukæmic cells transformed through the use of purine antagonists; the second relates to a use of combinations of anti-leukæmic agents in an attempt to suppress the selection of spontaneous mutations to resistance and dependence

All three variant sublines of leukæmia L1210, dependent upon 8-azaguanine (8-AG-D), resistant to 8-azaguanine (8-AG-R) and resistant to 6-mercaptopurine (6-M-R) show a striking increase in sensitivity to the folic antagonist Amethopterin. This change in response is similar to that recorded by Elion and Hitchings (1953) in a 6-mercaptopurine-resistant strain of *Lactobacillus casei* which shows a significantly increased requirement for folic acid.

adequate or that some metabolite other than the four physiological purines must be supplied. It will be of interest to attempt reversal of 6-mercaptopurine effects in leukæmic cells by the ribosides and ribotides of adenine.

Some suggestive preliminary data obtained through a study of nucleic acid metabolism of sensitive and dependent (8-azaguanine) leukæmic cells of leukæmia L1210 are at hand. 8-Azaguanine-2-^{14}C has been found to be incorporated into the RNA of sensitive leukæmic cells at levels 100 times the incorporation of this purine antagonist into dependent cells (Bennett, Skipper and Law, 1953). This may be considered as evidence that fixation of 8-azaguanine in nucleic acids may be related to the carcinostatic activity of the compound. Low levels of incorporation of the purine antagonist 2 : 6-diaminopurine (as 2 . 6-DAP-2-^{14}C) in dependent cells have also been found (Skipper, 1953) whereas the utilization of thymine and guanine (as 2-^{14}C products) is of the same order of magnitude in sensitive and dependent cells. Since 2 : 6-diaminopurine is known to be readily converted to nucleic acid guanine (Bendich *et al*, 1950) these results indicate a difference in capacity of the two types of cells to utilize this compound as a source of guanine.

The observations discussed above concerning metabolism of sensitive and transformed leukæmic cells suggest that differences in nucleic acid metabolism may exist.

It has been reported by Hirschberg *et al* (1952) and by Gellhorn (1953*a*) that experimental tumours most sensitive to 8-azaguanine have a low concentration of an enzyme (deaminase) capable of converting 8-azaguanine to 8-azaxanthine (a non-carcinostatic agent) in contradistinction to those tumours not influenced by the compound. Thus, it is suggested that the variation in response of neoplastic tissues results from their ability to metabolize 8-azaguanine to an inactive form. In preliminary studies comparing deaminase concentrations of L1210 sensitive and 8-azaguanine-dependent cells this does not appear to be true, since enzyme levels obtained, although relatively high, were the same in both types of cell (Gellhorn, 1953*b*).

Table X

EFFECT OF AMETHOPTERIN AND 8-AZAGUANINE GIVEN IN COMBINATION, EITHER SINGLY OR SIMULTANEOUSLY, ON SURVIVAL TIME OF TEST MICE BEARING ACUTE LYMPHOCYTIC LEUKÆMIA L1210

Experiment	No Mice	Dosage (mg/kg) Am	Dosage (mg/kg) 8 Ag	Survival Time in Days (range)	Percentage increase in survival	Remarks
Control	10	None		8·1 (8–9)		
Am – 8 Ag	10	3 × 5	75 × 9	17·0 (12–22)	112·5	
8 Ag – Am	10	3 × 5	75 × 9	18·6 (18–19)	129·6	
Am + 8 Ag (Simult.)	10	3 × 5	75 × 9	36·5 (20–90)	350·0	2 90-day survivors*
Control	16	None		7·6 (7–9)		
Am. – 8 Ag	9	3 × 4	75 × 8	19·2 (18–20)	152·7	
8 Ag – Am	9	3 × 4	75 × 8	20·1 (18–22)	164·5	
Am + 8 Ag (Simult.)	9	3 × 4	75 × 8	28·4 (19–90)	273·6	1 90-day survivor*
Control	16	None		8·1 (7–10)		
Am – 8 Ag.	8	3 × 4	75 × 8	20·0 (19–21)	146·9	
8 Ag – Am.	8	3 × 4	75 × 8	16·1 (15–17)	98·7	
Am. + 8 Ag (Simult.)	16	3 × 4	75 × 8	25·7 (11–90)	217·3	2 90-day survivors†
Totals (1) Control	42			7·9 ± 0·01		
Totals (2) Am – 8 Ag and 8 Ag – Am	54	(see above)		18·5 ± 0·33	134·2	Diff. (1) and (3) = 21·6 ± 4·0 $P < 0{\cdot}001$
Totals (3) Am + 8 Ag (Simult.)	35			29·5 ± 4·0	273·4	Diff. (2) and (3) = 11·0 ± 4·1 $P < 0{\cdot}01$
	131					

*All reinoculated at 100 days with *leukæmia L1210*, *died leukæmic* at 8, 10 and 10 days
†Negative leukæmia, *histologically*, at 180 days
Am – 8 Ag and 8 Ag – Am: compounds given singly in combination
Am + 8 Ag: compounds given simultaneously in combination

Table IX shows this striking difference in response of the dependent leukæmic cells (8-azaguanine) contrasted with the usual response of the sensitive leukæmia (Law *et al.*, 1953).

Table IX

8-AZAGUANINE-DEPENDENT LEUKÆMIA L1210 AND AMETHOPTERIN

Compound	*Dosage mg/kg*	*No Mice*	*Survival in days*
	8-Azaguanine-dependent		
None	—	68	15·8±0 45
8-azaguanine	75×8	74	12·1±0 23
Amethopterin	3×4–9	81	63 1±3·17*
	Sensitive		
None	—	145	7·9±0 09
8-azaguanine	75×8	56	12 4±0·20
Amethopterin	3×4–9	115	17 1±0 69

*39 mice (48 2 per cent) negative at 90 days

The data of Table X appear to provide a clear rationale for the use of two or more anti-leukæmic agents acting independently. The two most effective compounds used in the laboratory, Amethopterin and 8-azaguanine, have been shown to act as selective agents in the isolation of resistant and dependent mutants Each also has been shown to act independently of the other. Since there appears to be no known method for decreasing mutation rates, and it is unlikely that the host can alter the process of spontaneous mutation, the best approach appears to be an attempt to suppress the selection of spontaneously occurring transformations. The principle of combined therapy with two or more agents acting independently has been used successfully in infectious diseases, particularly in the treatment of tuberculosis. If the frequency of mutation to resistance of a cell, bacterial or cancerous, to drug A is 1×10^{-6}, and a frequency of mutation to drug B is 1×10^{-5}, only one cell in 10^{11} will simultaneously develop both mutations. Thus, doubly resistant mutants have a negligible probability of emerging in a sensitive tumour or bacterial population in the presence

Experimental evidence favours the assumption that the variant cells arise by spontaneous mutation, which occurs constantly in populations of leukæmic cells, the anti-metabolites acting as selective agents in the isolation and propagation of the variant forms.

Certain preliminary metabolic studies relating to mechanisms of resistance and dependence are given.

Some practical considerations relating to suppression of resistant leukæmic cells and the use of altered sensitivity to folic analogues are discussed

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of two or more effective agents which exhibit different mechanisms of action. It may be seen from the table that although these two antimetabolites, given one after the other at dosage levels below the MTD, are effective in increasing survival, when given simultaneously, there is a striking potentiation of effect. Many of these mice, though receiving a standard dose (8×10^5 cells) of leukæmic cells live beyond a ninety-day period and show no evidence of leukæmia. These survivors, in all probability, represent cases in which all or most leukæmic cells were killed and doubly resistant forms completely suppressed (Law, 1952*b*).

Summary

Transformations to resistance and to dependence are found to occur rather generally among acute lymphocytic leukæmias of the mouse. Two types of antimetabolites have been used in the development of these variant forms: folic acid antagonists and purine antagonists.

Leukæmic cells resistant to or dependent on folic analogues, are inhibited by other non-related anti-leukæmic agents, but show a characteristic cross-resistance (or cross-dependence) to all other 4-amino-substituted folic antagonists. Folic acid and citrovorum factor were found not to influence the growth of variant cells but both compounds reversed the growth-promoting action of Amethopterin in dependent leukæmic cells

Leukæmic cells resistant to or dependent on purine antagonists (8-azaguanine and 6-mercaptopurine) show cross-resistance (or cross-dependence) to all other purine analogues tested, but other non-related compounds remain carcinostatic. A striking increase in sensitivity to folic analogues of all anti-purine variants was observed.

The changes discussed are shown to be stable, irreversible and heritable No reversions to sensitivity or from one form to another have occurred among many lines carried in transplant for 50 generations or more.

I should like to make a few comments on this as it applies to clinical work and to some other experimental work. We have tried in Line I leukæmia in C58 mice to compare the effects of this tumour in converting folic acid to citrovorum factor with a resistant variant of the same line. As Dr. Law mentioned with his line, the L1210, we could find no difference between the resistant and sensitive cells, in contrast to the [illegible]

injections, they developed an alarming dermatitis So that there is a system in the skin which is very sensitive to this, at dosage levels which in our experience had no effect on the tumour whatsoever

I think the incorporation studies that Dr Law mentioned are ex- [illegible]

dependency

HADDOW: I also wondered about the speed of conferment of dependence

LAW This depends on the type of leukæmia selected. Sometimes it is rather rapid, in four to five transfers, in other cases it has taken 16–20 transfers

[illegible]

LAW You would expect so. But we haven't applied selective pressures to see if back mutations occur as they do occur in bacteria

[illegible]

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DISCUSSION

DUSTIN I should like to ask two simple questions. All these drugs inhibit growth of most cells, leukæmic and others Has any attempt been made to see if normal non-leukæmic cells would also become resistant to, or dependent on these drugs? May leukæmic cells have a higher mutation rate? Secondly, I agree that the drug fastness is apparently due to mutation. But might not these drugs be mutagens themselves?

LAW I see no reason why one couldn't get resistance or dependence in normal cells, it is just a very difficult thing to do I think that other cancer cells will do the same thing, I believe sarcoma 180 develops resistance to an antipurine It is also quite likely that some of these compounds are mutagens We have found, however, that if you start with a large population of leukæmic cells and select various sublines, that you arrive at resistance or dependence without the use of the compound.

BURCHENAL There is a fair amount of evidence in the literature about normal systems developing resistance, for example, mosquitoes and flies developing resistance to DDT, malarial organisms becoming resistant to antimalarials, and hamsters developing resistance over thousands of years to colchicine.

It has just occurred to me, after hearing Dr. Kieler, that these substances may be the equivalent of bacteriostatic agents they are cytostatic; *they are inhibiting the growth but they are not killing the cells. After incubation for a week or so with Amethopterin, he can still* transmit the leukæmia to mice With bacteria that is reasonably satisfactory—with a bacteriostatic agent the defensive mechanisms of the body will take care of the few that remain—but probably in human leukæmia we are dealing with no effective resistance on the part of the host. If we just inhibit these cells, perhaps even if we inhibit them with a combination of three or four agents, still they'll come back. I'm arguing *against myself, because I definitely believe in the rationale of combination* therapy

LAW Theoretically it is better to use agents simultaneously, if this is a spontaneous mutation in the genetic sense, than to use one agent followed by another agent—unless one encounters the situation which I discussed earlier where one agent changes the susceptibility of the leukæmic cells to another (e g. the antipurines increase the *susceptibility to the folic antagonists). That is a possibility.* But the situation in man is such that the agents are not very selective, there aren't very many of them, and by the time you give them to a patient you have a tremendous population of leukæmic cells You are very probably dealing with quite a large population of sensitive cells.

per cent or more was necessary in order to obtain a direct damaging

Moreover, Amethopterin had little influence on transplantability of sensitive and resistant cells. Even after incubation for one week with Amethopterin in a concentration as high as 100 mg. per cent, you could transfer the cultures to mice and they would all develop leukæmia and die in the course of seven to eleven days; and in this respect there was no difference whatsoever between sensitive and resistant cells.

We examined too the possibility of detoxification of the compound, using initial concentrations of 50 milligamma and 25 milligamma per ml in the cultures Even after six days' incubation no decrease could be demonstrated in the concentration of the drug in the medium by the

decrease of mitotic activity in the sensitive cultures, while a concentration of 100 mg. per cent was necessary to obtain the same effect in resistant cultures.

I wonder whether Dr Law has found any data on the intracellular distribution of the drug in sensitive and resistant cells? Counting the mitoses, we found that Amethopterin would not cause any shift in the distribution of the mitotic phases This is in disagreement with what has been found in fibroblast cultures but, as far as I understand, is in perfect agreement with Dr Dustin's findings. The absence of any phase specificity suggests that the action of the drug takes place during interphase, so it is conceivable that a decreased nuclear adsorption of the drug in the resistant cell may explain the resistance And if the intranuclear formation of citrovorum factor tends to overcompensate the neutralization of cytoplasmic citrovorum factor, a decreased nuclear absorption may even explain dependence

MOESCHLIN: In what way can we apply these findings to the clinical treatment of patients? In this experimental work we are studying a strain which already contains some resistant cells If we treat a patient, in many cases we have a non-resistant strain at the beginning and resistance develops after some weeks or usually some months. At present I think it is probably better not to apply the drugs in combination but to start with one drug and if resistance develops change to another one. I think we can prolong the patient's life longer than if we combine from the beginning.

BURCHENAL: With other drugs in leukæmia a resistance has also been shown, and the lymphosarcoma of Heilman and Kendall definitely

foci, and the marked tendency of the cells to invade the blood-vessels of the neoplastic area led us to suspect a relationship between the Murray-Begg endothelioma and certain types of leukosis.

We therefore sought to determine whether leukæmic forms of the Murray-Begg tumour could be induced, and in order to achieve this we tried to modify the host in some way. Experience has shown that every time a virus remains active in a modified host, changes in its behaviour are likely to occur. After many failures we have finally succeeded by utilizing X-rays. Chickens received whole-body irradiation of 1200 r one day prior to the inoculation of tumour cells, and the endothelioma which then developed was again grafted into irradiated chickens. In the third passage in irradiated fowls we obtained two tumours with leukæmia. The bone-marrow of one of these animals, injected intravenously, produced a tumour at the point of injection in the wing. One of the animals injected i v with a cell suspension of this tumour developed a pure leukæmia, without any tumour, the only case so far observed Intramuscular injection of the cells from the same tumour produced in successive generations six tumours with leukæmias in 26 fowls Thus far it has not been possible to maintain the process in its pure leukæmic form through i.v injection of leukæmic blood.

The leukæmic form of the Murray-Begg endothelioma is primarily characterized by the changes that occur in the blood. The picture shown in a blood smear is clearly that of leukæmic blood, the number of white cells is considerably increased, lying between 40,000 and 200,000. The new cells consist of large round or ovoid forms containing a large irregular nucleus, usually eccentric in position and having a delicate network of chromatin and several nucleoli. The cytoplasm, which is markedly basophilic, contains no granules but is frequently vacuolated. Mitotic forms are almost always found These cells therefore closely resemble hæmocytoblastic stem cells. Erythroblasts and megakaryocytes are sometimes present as well. Both the liver and spleen are markedly

LEUKÆMIC FORMS OF THE MURRAY-BEGG ENDOTHELIOMA

CHARLES OBERLING

WORK on the Murray-Begg endothelioma at our Institute, carried out for many years in collaboration with M. Guerin and Mrs. Lacour, has completely confirmed the earlier descriptions given by the British workers in 1930. The peculiar morphological features of this tumour readily distinguish it from all other filtrable fowl tumours. The cells present very variable aspects, which can be classified into three categories.

Firstly, there are the cells of the fibroblast-macrophage type. The zones of tumour that are composed of such cells may closely resemble a Rous sarcoma. Areas with abundant *macrophages may be found beside the fibroblastic regions*, and a striking feature of the Murray-Begg tumour is the abundance of giant cells, especially surrounding zones of necrosis.

The second characteristic type of cell is the round or ovoid basophilic cell which may be compressed into polygonal forms. Such cells always contain a more or less eccentrically-placed vesicular nucleus possessing a large nucleolus. Cytologically, *they closely resemble immature blood cells of* the hæmocytoblastic type.

The third class is composed of cells of endothelial type. These often occur when the tumour cells replace the endothelium of the vessels in the invaded areas, but it is considered that the tumour cell itself can give rise to endothelial structures independently of the nature of the tissue invaded, as shown by the formation of angiomatous structures.

Several considerations, such as the presence of the hæmocytoblast-like cells, the tendency of the tumours to give rise to diffuse infiltrations rather than well-defined metastatic

obtain leukæmias from a process which for thirty years has always been known as a sarcoma.

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DISCUSSION

FURTH We also described a virus (our No 2) which caused both

When we injected the virus subcutaneously we got sarcomas, and when

known as a tumour, and now it appears that under certain circumstances this tumour can take the form of a leukæmia

increased in size—the latter weighing 5–8 g.—and are heavily congested and show the violet colour typical of erythroblastosis. The lungs are infiltrated with hæmorrhagic areas, and the kidneys similarly may show dark infiltrations.

Microscopically the liver shows infiltrations, mostly located in the periportal spaces. Hepatic cells have disappeared from the affected areas, and are replaced by immature elements in the form of continuous sheets in which the individual cells are not easy to distinguish.

In the bone-marrow and spleen, the normal parenchyma is replaced by infiltrations of large cells identical in all respects with those found in the circulating blood. Even at low magnifications these cells are recognizable by their vesicular nucleus and large nucleolus

The same cellular infiltrations are found in the lungs, often massively developed along the interlobular divisions, where all trace of lung tissue may be obliterated.

In the kidney these foci fill the dilated vessels and intrude into the interstitial tissues, thus destroying the parenchyma.

Controls were represented by the animals of the normal tumour transfers Suddenly leukæmias occurred in these chickens too So far, four cases have been observed in 21 fowls injected with tumour material.

It must be added, however, that at a certain stage the original tumour strain had been lost and replaced by one which had passed through an irradiated chick. In order to make sure that the appearance of leukæmic forms is really related to the X-ray action and not due to a spontaneous variation, this work has to be started again with the original strain of Murray-Begg endothelioma. This investigation is now under way.

Whatever may be its result, there clearly exists a close relationship between Murray-Begg endothelioma and leukæmic conditions. It may therefore be concluded that not only is it possible to derive sarcomas from leukæmia, as was shown by Oberling and Guerin some twenty years ago, but also to

not suitable for the electron microscope, where the greater power of separation reveals more subtle alterations. The idea of artifact has been developed· we are no longer content merely to try to conserve the broad lines of tissue and cellular structure, but wish to immobilize the macro-molecular structures. This problem has been the object of some recent studies (Policard *et al.*, 1952, 1953) to which we refer the reader.

The necessity of having very thin preparations is another difficulty, the final solution of which is not yet apparent. The method of making sections has not yet been perfected. The first really encouraging results date from only a few months ago (Sjostrand, 1953) It has thus been possible to [illegible]

and enzymic or chemical digestion techniques (Bessis, 1954).

These techniques have provided information on some cellular constituents (e g. the number and form of cytoplasmic granulations), but have shown nothing definite, or nothing new, about the disposition *in situ* of even the largest protein molecules, nor about the structure of the nucleus, for instance. The first escapes us because of fixation artifacts, the second because of the thickness of the object examined. In short, the enormous resolving power of the electron microscope cannot yet be utilized. The apparatus can magnify 100,000 times, but in the tissue we are considering, it reveals mainly artifacts. We may, however, hope that improvements in the near future will reverse that judgment

Investigators have attacked the problem of the morphology of the leukæmic cell with the electron microscope from two different directions· the search for a virus, and for sub-microscopic malformations of the cellular constituents; we shall summarize their observations.

Search for a Virus

As is well known, many viruses have been made visible with the electron microscope, not only in the isolated state,

RESPECTIVE VALUES OF ELECTRON AND PHASE-CONTRAST MICROSCOPY IN THE STUDY OF THE LEUKÆMIC CELL*

A. LACASSAGNE and M. BESSIS

STUDY of the leukæmic cell with the aid of the classical histological and hæmatological techniques does not seem capable of much further development. This is the reason for the interest shown during the past ten years in new methods of investigation, especially in cytochemistry and electron and phase microscopy. We shall try to summarize the results obtained by the latter two techniques and to discuss the limits and possibilities of their application.

Electron Microscopy of Blood Cells

The electron microscopes at present avail[illegible] magnification of about 100,000 [illegible] is limi[illegible]
[illegible] [illegible] 10 mm. of mercury; t[illegible] are dead and completely desiccated. The other arises from the impossibility of examining objects more than about 0.2μ thick.

These factors explain why the very considerable advances made in the study of bacteria, viruses and macro[illegible] have no equivalents in cytolo[illegible] we are [illegible] befo[illegible] histological section and [illegible]

The necessity of observing dead and dried cells introduces the difficult question of fixing. Those fixatives which are considered suitable for the optical microsco[illegible]

*In [illegible] film [illegible]

normal cells of the same type and at the same stage of maturation. A leukæmic myelocyte or myeloblast can only be compared with a normal myelocyte or myeloblast, and not, as has been done, with normal granulocytes. It is difficult, however, to separate and prepare for the electron microscope immature cells present in small numbers in the spinal marrow and mixed with all kinds of other cells. Moreover it is difficult to recognize the granulations (mitochondria, azurophilic and specific granules, etc.) scattered in the cytoplasm without the aid of their staining affinities.

We shall confine ourselves here to indicating the findings which can be definitely said not to be artifacts.

Myeloblastic leukæmia. The cells of these forms of leukæmia have the property of spontaneous spreading on certain carriers. Their hyaloplasmic veil is very thin and can therefore be examined directly by transparency.

Using this technique, Oberling and co-workers (1950), and Bernhard and co-workers (1950) observed, as we mentioned earlier, minute elements of which the smallest measured about

these formations were to be found in normal blood cells and could even reach a considerable degree of development there (Bernhard *et al.*, 1950).

In point of fact, for demonstrating the form and true number of granules contained in the cytoplasm, other techniques of investigation are preferable to spreading, which only allows the organizers present in the hyaloplasm to be seen (Bessis and

visible only under the electron microscope, it is difficult to do more than speculate. In the present state of technique, the first necessity is to explore and identify all the shapes and sizes of granulations which may be encountered in normal myeloblasts—*and it is certain that this work will take many years.*

but also within the cells in certain special circumstances. Some investigators have, therefore, turned their efforts in this direction. They have been encouraged by the study on Rous sarcoma cells by Claude, Porter and Pickles (1947). In the hyaloplasm of sarcomatous cells these authors discovered the presence of spherical granules of about 70 mμ in diameter, extremely opaque to electronic radiations, not found in non-sarcomatous control cells. Previous ultra-filtration and ultra-centrifugation studies (Claude, 1937; 1939) had shown that the infective particle must have precisely these dimensions. *These observations were therefore loudly acclaimed.*

In 1950, Oberling, Bernhard and collaborators described similar granulations in human leukæmic cells, and put forward the hypothesis that this was the leukæmic virus. *Shortly* afterwards, however, it was admitted that comparable appearances were to be found in normal cells (Bernhard *et al.*, 1950; Bessis and Bricka, 1951).

We may, therefore, say that the search for a virus in leukæmic cells has for the moment received a set-back. This does not imply, however, that it is not there; the virus might be concealed (in the nucleus, for example), it might pass through phases in which it forms part of the host-cell proteins, and is therefore unidentifiable, or, as Bernhard and Oberling (1953) suggest in the case of the Rous sarcoma, it might only be present in one cell out of 100 or 1,000. It might also be represented by one of the numerous sorts of granulations of all shapes and sizes found in the cytoplasm of leukæmic cells.*

Search for Characteristic Malformations

We must first of all refer to the difficulties encountered in finding controls *Leukæmic cells must be compared with*

*ADDENDUM There is no reason *a priori* for thinking that the virus should be any more opaque than other cellular proteins to electronic rays With regard to the Rous sarcoma, for instance, Claude's studies in particular have shown that the virus has a molecular weight and chemical composition very close to those of the ribonucleoprotein microsomes of normal cellular protoplasm It is difficult to see why one should expect to be able to distinguish the virus in cancer cells from other cytoplasmic particles of identical composition and molecular weight

and not myeloblasts, and in chronic leukæmia, while Auer bodies characterize acute leukæmia. In addition, these rods appear to be formed during the crushing. In this, and this only, they resemble Charcot's crystals. Finally, they are found only in some lymphocytic leukæmias, and never in myeloblastic or monocytic leukæmias; on one occasion, however, some were observed in a plasmocytoma.

In conclusion, the electron microscope has shown in leukæmic cells: (1) malformations or immaturities of the cellular components conforming to classical ideas on leukæmic cells; (2) para-crystalline formations which are not constant but apparently specific for the type of cell affected.

Study of Leukæmic Cells by Phase-contrast

In the ordinary optical microscope, little can be seen in living protoplasm; the differences in refraction between its various structures are too slight, and one must be content with the more or less indistinct image obtained by stopping down the condenser to the limit. On the other hand, the phase-contrast microscope gives a distinct image of the different cellular components chromatin, mitochondria, centrosome, specific granules are all often clearer than in fixed and stained preparations. It thus becomes possible to observe signs of damage or pathological states directly in the cell; in short, to observe the effects of experimentation on the living cell

The importance of phase-contrast examination is further increased by combining it with microcinephotography. Many movements of cells and within cells when seen with the optical microscope are so slow as to pass unnoticed. Time-lapse cinephotography allows them to be recorded and analysed. Tracings obtained from these pictures permit the reconstruction of these phenomena and the measurement of deformities undergone by various cellular organs as a function of time The number of reports on this subject is still very few (Policard and Bessis, 1952, 1953; Chevremont and Frederic,

Among all the granulations to be seen in normal and leukæmic myeloblasts, some are visible under the optical microscope and are perhaps characteristic of leukæmic myeloblasts; these are Auer bodies; their appearance is para-crystalline, rod-shaped, more or less slender lozenges, measuring about 0·5 to 7μ across. The electron microscope shows them very plainly. It also shows the existence of "micro-crystals" (Bessis, 1951) invisible under the optical microscope, and these may be found in cells without any large crystals. As these formations have so far only been observed in leukæmic cells, they certainly deserve further study.

Myelocytic leukæmia. With the aid of the electron microscope, details have been obtained about malformations of cells, especially the granular anomalies, previously described with the optical microscope. They vary greatly in numbers from one cell to another· some cells have almost none at all; others are practically full of them. The form of the granulations themselves is very much modified; some are twice or three times as long as usual. Moreover, the majority of the cells present very few rod-shaped granulations and large numbers of round ones, which is the reverse of normal. This probably represents the survival of immature granulations in cells with mature nuclei, and is just one of the forms of anarchy in nucleo-cytoplasmic development.

Lymphoblastic and lymphocytic leukæmia. As the cells of lymphocytic leukæmia lack the ability to spread, they have so far only been observable by grinding and destruction methods The latter technique has permitted curious formations to be observed (Bessis, 1951) Among the mitochondria have been observed rods with rounded ends, numbering from two to 20 to a cell, the largest measuring from 1 to 1·5μ in length by about 0 2μ across, and the smallest being considerably beyond the resolving power of the optical microscope The rods are sometimes arranged parallel and very close to each other. These formations cannot be likened to Auer bodies; they are found in lymphocytes

succession of different morphological phenomena leading to disintegration of the cell seems to take place according to quite typical modalities, of which there seem to be five different varieties: fragmentation, karyolysis, pyknosis, formation of exoplasmic bullæ (potocytosis) and vacuolization. These different alterations certainly correspond to classical anatomo-pathological aspects; but here one may see them actually taking place before one's eyes, measure the degree of inflation or retraction of the structures, the order of precedence of the phenomena, etc.

It is particularly interesting to know that different drugs or toxins produce only one or other of these aspects in the cells. Studies on this last point are still only at their very beginning.

We have undertaken a study of the action of certain anti-leukæmic drugs, polonium radiations and anti-leucocytic antibodies on leukæmic cells We report here our first results in brief.

Polonium. From a silver film on which polonium has been deposited, fragments measuring 0 5 to 10μ are introduced between slide and coverslip The effect of alpha-radiation is already visible after a few minutes The cell begins to swell, it seems to imbibe liquid, the cytoplasmic granules are animated by lively Brownian movements At this stage the nucleus is still normal or slightly diminished in volume. In a second period, after about five or six minutes, the nucleus becomes lighter and considerably enlarged. The nucleoli then appear quite clearly dark against the light chromatin ground. Often they divide into three or four parts. Finally in most cases the nuclear membrane bursts and the nuclear contents and the nucleoli are spilled out into the cytoplasm, some ten to fifteen minutes after the beginning of the experiment.

Hypotonia. Very similar appearances are obtained by the simple action of distilled water This suggests that the cell alteration observed in instantaneous death due to radiation is due to the breaking down of a barrier of osmotic equilibrium.

1951), but everything points to its being destined to play an important part in cytological research.

We shall now briefly review the principal new discoveries made with the phase-contrast microscope on the leukæmic cell with or without cinematographic recording.

Appearance of Different Leukæmic Cells

Phase-contrast shows up well the nucleus, nuclear membrane, nucleoles, specific granulations, mitochondria and various cytoplasmic formations (fat vacuoles etc.). The Auer bodies in acute myeloblastic leukæmia are much better demonstrated than by staining methods (Bessis, 1949).

Microcinephotography allows the various cell-movements (reptation, spreading, putting out various expansions) and intracellular movements (oscillation of the centrosome, formation of contractile vacuoles, etc.) to be studied (Policard *et al.*, 1952, 1953; Bessis and Locquin, 1950). The cell-movements are to a certain extent characteristic of the different cell types, and sometimes permit the diagnosis of the type affected when no clear distinguishing feature can be discerned in fixed and stained cells. In particular, monocytic leukæmia may be differentiated from certain acute myeloblastic or lymphocytic leukæmias.

After a certain time between slide and coverslip, a transformation is often observed in the nucleus, which, from a regular rounded shape, becomes folded and progressively takes on a three- or four-lobed appearance The nucleoli often divide in the same way, each in one of the nuclear lobes. Here the so-called "Rieder cells" are being formed under our very eyes. These cells are in no way specific for leukæmia, as has been maintained. They may be seen forming in normal blood or bone marrow under the same conditions. They undoubtedly represent non-specific signs of cell pathology, but it is true that they are often found in leukæmic cells *in vivo*.

Another interesting aspect of the behaviour of normal and leukæmic cells *in vitro* may be deduced from the changes preceding cell death. During spontaneous autolysis, the

reactions of various cells to environmental modifications and the administration of various anti-leukæmic drugs. Substantial results may be expected from it in the near future.

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DISCUSSION

GORER Can you tell us any more about the case with auto-immunization?

BESSIS It was a woman with aleukæmic lymphatic leukæmia

Antibodies. Heterogenic antibodies (anti-human-leucocyte rabbit antibodies) produce two types of phenomena according to their strength. Very active antibodies lyse the cells in a few minutes, and a sequence of phenomena the same as that just described is observed. Less active antibodies cause granulocytic phagocytosis of the cells A regular pursuit of the sensitized elements by healthy cells takes place, usually ending in complete inclusion or the separation and phagocytosis of only a part of the cell (a phenomenon identical with erythrophagocytosis Policard and Bessis, 1953).

In one case we were able to study cinematographically the action of an auto-antibody. It was in the case of a woman (André *et al.*, 1954) with lymphoid leukæmia, and with appearances of lymphogranulomatosis in the spleen. Homogenous and autogenous lymphocytes were destroyed and phagocytized by her serum. The pursuit of lymphocytic elements by the granulocytes was particularly striking in this case.

Aminopterin. Traces of this folic acid antagonist, added *in vitro*, produced violent contractions of the cytoplasm and of the nucleus especially, terminating in tortured appearances. Two- and three-lobed forms are frequent. Methopterin produces similar results, with teropterin (inactive against leukæmia) no such appearances are observed These signs of cell pathology are, however, in no way specific for these drugs, we have observed them with quite different products, such as colchicine or phenobarbital.

Summary

The electron microscope has revealed some new malformations in leukæmic cells, but it has not yet been possible to find anything specific Great technical difficulties in the preparation of objects make the use of very high-power magnification pointless at the moment.

The phase-contrast microscope has shown many new details in the dynamic appearance of leukæmic cells. Combined with cinematography it facilitates the study of

PATERSON. Have you seen the effect on the dividing cell as well as on the resting cell?

BESSIS: No, in this experiment we only studied the immediate lethal effect.

D
polc
dist
ther
break. With polonium you have what you find after X-rays, the chromatin first becomes very dense against the nuclear membrane which eventually breaks

BESSIS. That depends on the rapidity. When the cell destruction is rapid, the chromatin has no time to condense before the nucleus breaks. When the process is slow the chromatin sticks to the nuclear membrane and the nucleus becomes "pycnotic". The same effect is produced by many other noxious agents, as, for example, Aminopterin or Methopterin.

DUSTIN I think the question of the Auer bodies is rather important. Of course, they are not viruses, but they may be some kind of abnormal protein built up by viruses So it is very important to know if they exist only in leukæmic cells Dr Bessis has stated very definitely that they do. But I feel sure I have read somewhere that Auer bodies have been described in non-leukæmic cells.

MOESCHLIN: A Japanese published a monograph in 1938 or 1939 on the Auer bodies (Rohr has the original paper) He could demonstrate them in normal lymphocytes, in monocytes and in neutrophils, and he has wonderful pictures, so I think there is no doubt. But whether they are chemically different from the Auer bodies in leukæmia is another question.

BESSIS The old hæmatologists used to call lymphocytes some cells which we now call myeloblasts To my knowledge, Auer bodies have been described only in granulocytes and only in leukæmias. Other crystalline bodies have been described in plasmocytic (Undritz) and in lymphatic leukæmias (Bessis).

She had severe transfusion shock, and then could not be given further transfusions, although we had very carefully selected the blood. We decided to perform a splenectomy in an attempt to stop the hæmolytic process. The spleen was enormous. To our surprise, when the sections were made, we found Sternberg cells. We thought that it was an association of Hodgkin's disease and lymphatic leukæmia, which is very rare. The titre of the antileucocytic auto-antibodies was much reduced afterwards. Now, after three months, she is better and she has no hæmolytic anæmia, but still has leukæmic symptoms.

GROSS: Was this woman sensitized by transfusion?

BESSIS: Yes, it is possible.

DUSTIN: *Have you studied the formation of "L.E. cells" by the microcinephotograph technique?*

BESSIS: Yes, we saw the formation of "L.E. cells" and "Tart cells" in all stages. When the cell is lysed and then phagocytized we get an L.E. cell. When the cell is phagocytized without having first been lysed one gets the Tart cell. In the case of the patient I have just mentioned both phenomena could be observed.

POLLI: Can you say from your experiments that the nuclei of the leukæmic cells differ from normal ones?

BESSIS: I do not know.

FAGRAEUS: How did you carry out the experiment with rabbit antibodies?

BESSIS: *The antileukæmic cell serum was obtained by inoculating* rabbits with human leukæmic cells. The serum was then subjected to adsorption by red cells to eliminate red cell antibodies.

ISRAELS: *Do polymorphs phagocytize lymphocytes in normal blood,* or is this only seen in leukæmic blood?

BESSIS: Polymorphs phagocytize everything, red cells, platelets, etc., *if the cells are sensitized by antibodies.*

MOESCHLIN: Dr Bessis speaks of auto-antibodies. I think it would be better to use the term *iso-antibodies* to normal cells, because probably *they are not real* auto-antibodies, they are probably non-*specific* antibodies which are adsorbed on the surface of the cells. I think true auto-antibodies are very rare.

BESSIS: But in the case of our patient, *she phagocytized her own cells.*

MOESCHLIN: Yes, but you may have, for example, a virus, or a non-specific antibody which is fixed on the cell surface and changes the cell *in such a way that it is regarded as a dead or foreign body which will* then be phagocytized. It is just a matter of definition, but I think auto-antibodies are real antibodies which are only specific against their own cells, but *these iso-antibodies are only specific for some* other kind of antigens. A typical example is the acquired hæmolytic anæmia and sometimes *even* pancytopenia (see Moeschlin and co-workers, *Blood,* 1954), *in "mononucleosis"* or in "atypical virus pneumonia".

PATERSON: What was the dose of polonium salts, and of irradiation?

BESSIS: We put the polonium on silver, and placed the little particles *of silver (0.5–10 micra)* in the medium. It is impossible to say what the individual dose was.

and the leukæmias are classified into (a) thymic, with or without generalized leukæmia, and (b) leukæmias other than thymic. The thymic leukæmias were almost invariably lymphoid; the others lymphoid or of the reticulum cell type.

The number of animals surviving thirty days and received by us for observations on late effects (shown in Table I)

Table I

THIRTY-DAY MORTALITY OF MICE EXPOSED TO ATOMIC DETONATION

Dose, r	*Number exposed*	*Number dead*	*LD Per cent*
870–932	440	431	97 9
812–841	440	385	87 5
759–785	440	268	60 9
711–733	440	130	29 5
631–687	440	41	9 3
491–556	440	17	3 9
(367–424	440	254*	57 7*)
287–318	440	14	3 2
192	220	6	2 7
0	620	7	1 1

*These figures are incomplete due to loss of animals during transportation from field laboratories.

indicate that the midlethal dose was about 750 r. All further data will refer to this population surviving the atomic detonation by at least thirty days. Fig 1 indicates that 192–556 r, which virtually killed no animals within thirty days, reduced longevity in all dose groups, the degree of reduction was proportional to the dose in the range of 841–491 r but *not in the lower dose range*

Longevity data are essential in the analysis of the incidence of leukæmia and tumours. The thymic lymphomas of mice are the first to appear, the nonthymic lymphomas, reticulum cell sarcomas, and myeloid leukæmias have a longer latency period Early death, from any cause, may eliminate potentially leukæmic animals. Among the various tumours those of the pituitary have the longest latency period (over fifteen months). The possible relation of pituitary tumours to leukæmias will be considered later

INDUCTION OF LEUKÆMIA BY IONIZING IRRADIATION*

J. FURTH and A. C. UPTON

SINCE Jagic *et al.* described (in 1911) four cases of leukæmia among radiologists and one in a radium worker, the increased incidence of leukæmia among people exposed to ionizing radiation has been the subject of numerous articles. This causal relation was first confirmed experimentally by Krebs *et al.* (in 1930) and later by many other investigators. These reports have been fully reviewed elsewhere (Furth and Upton, 1953*a*; Furth and Lorenz, 1954). In this paper we propose to survey relevant recent studies of our own and to discuss problems of leukæmogenesis on the basis of these and other investigations.

Leukæmia Induction in Mice by Exposure to Experimental Nuclear Detonation

Over 4000 genetically uniform mice (LAF_1), six to twelve weeks of age, were exposed to atomic detonation at various distances so as to receive an estimated 192 r to 932 r. The radiation was composed predominantly of gamma rays of high energy with some neutrons. The dose lethal to 50 per cent of the animals was about 750 r Subsequent to exposure, the mice were individually caged until natural death and kept in the same air-conditioned room, the cages being randomly distributed. The present analysis was made thirty months after exposure, when a small number of animals were still alive. Each dose group originally contained 110 male and 110 female mice; the controls numbered 300 of each sex. For simplicity of presentation adjacent dose groups are combined,

*Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission

induction of mediastinal lymphoma (cf. Kirschbaum, 1951). An understanding of the opposite responsiveness of mice of the two sexes to the development of thymic lymphoma would call for a special study involving irradiation of castrates of both sexes of the respective strains and the administration of sex hormones in parallel series.

The cumulative incidence of nonthymic leukæmias is shown in Fig. 3 A final interpretation of these data will

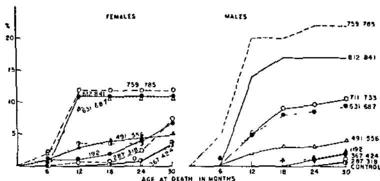


FIG 2 Cumulative incidence of thymic lymphoma in mice exposed to nuclear detonation, expressed as percentage of those surviving 30 days' post-irradiation

have to be postponed until histological study of this heterogeneous group has been completed and all data placed on punch cards, enabling a correlation of survival time and incidence of diseases of various types It can, however, be concluded that lymphomas other than thymic occur later and at a lower rate than thymic lymphomas, and it appears probable that they develop earlier and with somewhat increased frequency among the animals exposed to ionizing irradiation than in the controls

The human counterpart of the total-body irradiation of animals is the unfortunate exposure of people to atomic explosion. The reports of Folley *et al.* (1952) indicate a marked

Contrary to earlier findings, the mortality rates were greater in females. This could be due either to a strain difference or to individual caging, which prevented fighting, a common cause of intercurrent death among the males.

The cumulative incidence of thymic lymphoma (shown in Fig. 2) indicates that, again contrary to earlier findings, males had a higher incidence of thymic lymphoma at high dose-levels than females and that in males there was a fair

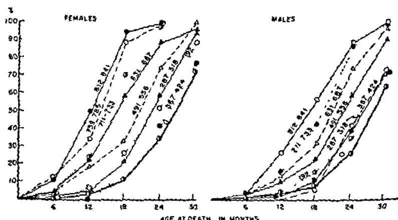


FIG 1. Cumulative mortality of mice exposed to nuclear detonation, expressed as percentage of the number surviving 30 days' post-irradiation The figures on the curves express the exposure intensity in r units

correlation between dose of radiation and lymphoma incidence. The disease occurred with greatest frequency in animals six to twelve months of age, but as many as 5 per cent of the males in the dose group above the LD_{50} had thymic lymphomas at six months. It is notable that at eighteen months this type of lymphoma was still absent in non-irradiated males.

It is known that œstrogens are leukæmogenic in certain strains of mice (Gardner, Dougherty and Williams, 1944) and that this effect can be nullified by androgens (cf. Kirschbaum, 1951). Œstrogens are highly synergistic with X-rays in the

have been myeloid and have occurred mostly in young persons, the peak incidence being in the second decade (Lange *et al.*, 1954).

Most radiation-induced leukæmias of man are myeloid. The leukæmias induced in the strain exposed to nuclear detonation, as in most other strains of mice, are lymphoid and predominantly of the thymic type. Different strains of mice vary in their responsiveness to ionizing irradiation. Mice of the RF strain, when exposed to ionizing irradiation, develop myeloid leukæmia with great frequency, as will be described presently.

Exposure of RF Mice to Ionizing Irradiation

In a large-scale study carried out in 1949–52 mice were exposed in the thermal column of the Oak Ridge reactor to quantities of ionizing radiations ranging from the LD_{50} dose to 1/16 of the LD_{50}. The neutron flux was approximately $0{\cdot}9 \times 10^{9}$/cm.^{2}/sec. and the gamma contamination estimated to average 5·9 r/min The inability to eliminate gamma rays or to evaluate them precisely, makes it difficult to estimate the relative biological effectiveness of slow neutrons. For comparison, siblings were exposed to doses of X-rays equalized on the basis of their acute lethal effects In so doing it became evident that the induction rate and types of leukæmia were about the same for a given dose of either type of irradiation. Since the results, already detailed elsewhere, did not disclose significant qualitative differences between neutrons and X-rays with respect to leukæmogenesis (Furth and Upton, 1953*b*), we are presenting the combined results to illustrate the induction of myeloid leukæmia by ionizing irradiation in the RF strain (Fig. 4).

Myeloid leukæmia is induced with greater frequency in the males of the RF strain than in females One hundred and twenty-eight roentgens appears to cause more myeloid leukæmias in both sexes than 512 r This can be explained by the fact that thymic lymphoma, also induced in this strain by 512 r but not by 128 r, appear earlier than myeloid leukæmia,

increase of leukæmia in the population exposed to atomic bomb irradiation at a distance below 1500 m. of the hypocentre of the explosion, the incidence rate decreasing with the distance.

The more recent report of Lange *et al.* (1954) gives the following percentage figures of leukæmia incidence:—

Under 1000 m.	0·7 per cent
1000-1499	0·2 per cent
1500-2499	0·02 per cent
Over 2500	0·006 per cent

FIG. 3 Cumulative incidence of nonthymic leukæmia in mice exposed to nuclear detonation, expressed as percentage of those surviving 30 days' post-irradiation.

Most people within the 1500 m. radius exhibited symptoms of radiation sickness; thus, the leukæmia-inducing dose in both men and mice is relatively high following a single exposure. The findings of Lange *et al.* indicate that the rate is now on the decline, the peak having been reached in 1950 (21 cases) and 1951 (19 cases). In 1952 11 cases occurred. There is, however, no way of telling how long the incidence rate will be elevated and whether or not another peak will occur. Thus far, most induced leukæmias of this population

The Effects of Cortisone on the Development of Leukæmia in Mice

Much current work on the induction of leukæmia is related to thymic lymphoma. The spontaneous leukæmias in the high leukæmia strain AK and in most other strains reported are predominantly thymic; similarly, the leukæmias induced by irradiation are predominantly thymic (Furth and Furth, 1936). Removal of the thymus prevents both the development of spontaneous lymphoma (Furth, 1946) and its induction by ionizing irradiation (Kaplan, 1950).

Involution of the thymus has long been known to follow stressful diseases, and the great variations in the incidence of thymic lymphoma in AK mice have been explained by accidental involution of the thymus. When cortisone became known as the hormone mediating thymic involution, experiments were undertaken on the effect of cortisone on leukæmia incidence by three groups of workers, apparently independently. Woolley and Peters (1953) studied the effect of administration of cortisone on spontaneous leukæmia in a high leukæmia strain, Kaplan *et al.* (1951) on leukæmia induction in a low leukæmia strain, and Upton and Furth (1954) studied both

Fig. 5 records two sets of experiments; in one a single set of three injections (3 × 1 mg. cortisone) was given on three consecutive days to mice of the high-leukæmia strain AK, in the second three sets of such injections were given. The induction of spontaneous leukæmia was inhibited but not prevented by this treatment Woolley and Peters gave cortisone over a longer period of time and caused a more marked inhibition of leukæmia development.

Fig 6 records the results of a set of experiments in which the effect of cortisone on the induction of leukæmia was tested in the RF strain. A single exposure to 350 r proved highly leukæmogenic in this strain and both thymic lymphomas and myeloid leukæmias were induced, as already indicated in Fig 4. Cortisone alone failed to produce leukæmia.

and by the assumption that many mice susceptible to the induction of thymic lymphoma would also have developed myeloid leukæmia had they not been killed by the former. The relation of dose to myeloid leukæmia incidence in this strain is being tested further by eliminating development of thymic lymphoma by irradiation of thymectomized animals.

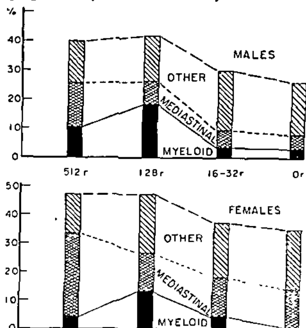


FIG 4 Combined incidence of various types of leukæmia in mice exposed to thermal neutron or to X-radiation The r's refer to 250 kv X-rays and to biologically equivalent doses of slow neutron-gamma radiation Leukæmias designated as "other" include generalized lymphomatosis and reticulum cell sarcomas.

With respect to induction of lymphoid leukæmias, the behaviour of the RF strain is characteristic in that thymic lymphomas *are induced with much greater frequency in* females than in males and in that nonthymic lymphoma occurs with great frequency in old controls. In non-irradiated males the total incidence of lymphoma may equal that in the irradiated male.

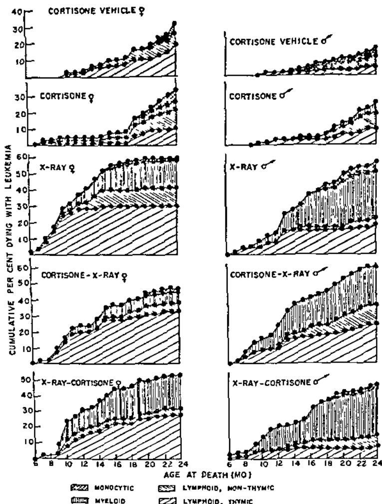


FIG 6 Cumulative incidence of leukæmia of various types in RF mice irradiated at 8–12 weeks of age Cortisone, 1 0 mg on three successive days, was given intramuscularly one week before or 2–3 weeks after 350 r of whole-body X-radiation. Cortisone vehicle was given simultaneously in comparable quantities

A marked reduction of mediastinal lymphoma occurred only in males in which cortisone treatment followed irradiation, but even this is not highly significant ($P \sim 0 \cdot 09$). In the group of females X-rayed after cortisone treatment, the myeloid leukæmia incidence was much lower than in controls, but this too is of questionable significance ($P \sim 0 \cdot 12$).

In these experiments cortisone and then X-rays were given

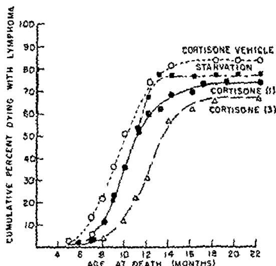


FIG 5 Cumulative incidence of lymphoma in AKR males given cortisone, 1 0 mg intramuscularly, on three successive days, of cortisone vehicle (1 *per cent* benzyl alcohol in 0 9 *per cent saline*) in comparable dosage (1) refers to a single series of injections given at 8–16 weeks of age, (3) to three such series of injections given at 0, 16 and 23 weeks of age, respectively. Starvation was done by withholding food for seventy-two hours when cortisone (3) was administered

one week apart. In earlier studies on co-leukæmogenesis with X-rays and methylcholanthrene there was a marked enhancement of leukæmogenesis when these two agents were given one week apart. Preliminary experiments have shown that one week following 350 r of X-irradiation there is regeneration of lymphoid tissues, with greatly increased mitotic activity. Recently Kaplan and Brown (1952) have shown

cells after irradiation, brought about by diverse agents, lessens the likelihood of late effects of irradiation, be they cataracts or leukæmia.

5. Irradiation may not in itself lead to a leukæmic change but may merely make cells sensitive to other agents, such as hormones, which may minimize or "realize" leukæmic transformation of cells altered by irradiation. There is ample analogy for such a sequence of events in the field of co-carcinogenesis.

6. A possible new factor in the induction of lymphoma by irradiation became evident when it was discovered that many irradiated animals developed pituitary tumours and that most of these neoplasms secreted ACTH (Upton and Furth, 1953, Furth, Gadsden, and Upton, 1953) It seems that depression of adrenal function may have played a major rôle in the induction of these tumours. Atrophy of the adrenal cortex in irradiated animals is a common finding that has thus far not been given adequate attention. It is known that the incidence of thymic lymphoma is greater in adrenalectomized than in normal animals

ACKNOWLEDGEMENT

The co-operation and help of many members of the Atomic Energy Commission and the Armed Forces, to be named in final publication, is gratefully acknowledged

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that when a given dose is fractionated at weekly intervals, the leukæmogenic effects are greatly enhanced. It is noteworthy that cortisone appears neither leukæmogenic nor co-leukæmogenic under similar conditions.

General Considerations

1. All types of ionizing radiations, electromagnetic and corpuscular, have proven to be leukæmogenic, and the available data suggest the generalization that there are no basic qualitative differences in leukæmogenic ability of different types of radiations.

2. There are marked species and strain differences with respect to susceptibility to leukæmia induction by ionizing irradiation—man and the mouse being sensitive; the rat, guinea pig, and rabbit resistant—but species of animals other than the mouse have not been adequately studied.

3. All types of leukæmias can be induced in mice; that most readily induced and extensively studied is thymic in origin. Induction of thymic lymphoma is subject to profound hormonal influences, adrenal and androgenic hormones inhibiting and œstrogenic hormones enhancing it in certain strains. In one strain studied thymic lymphoma was induced with greater frequency in the male. There is inadequate knowledge of how lymphoid leukæmia other than thymic is affected by these hormones, and the little evidence available suggests that myeloid leukæmia does not react similarly, if at all, to these hormonal influences.

4. The pathogenesis of leukæmia induction is unknown. A basic alteration appears to take place in the lymphocytogenic reticulum cells. It seems that agents which cause leukæmia operate not by an action on mature lymphocytes, which live only a few hours, but on the lymphocytogenic reticulum. Accordingly, a mere transient atrophy of the thymus, caused by cortisone, has but little effect on leukæmia development. The nature of the alteration in the lymphocytogenic reticulum caused by X-rays which makes it produce malignant lymphocytes is the essence of the problem. Hastened recovery of

Furth: By supposing that all neoplasms in irradiated animals are virus-induced, you would carry the argument *ad absurdum*. For example, almost every irradiated female mouse develops ovarian [illegible]

[illegible]

probably also many viruses, without necessarily showing symptoms of disease. It is quite possible to consider that oncogenic viruses, existing mostly in a latent form, are extremely common

Lorenz: There are some remarkable differences between acute and chronic irradiation in these mice While Dr. Furth finds with single exposures that males get more thymic lymphomas than females, in chronic irradiation when we give 8 8 r, eight hours per day throughout the entire life span, we get more lymphomas in the females Now it may just be that our parent strains are somewhat different In chronic irradiation the lymphomas do not come up until about fourteen months [illegible]

[illegible]

strains and also hybrid animals We have a leukæmia incidence of about 4 per cent in both irradiated and control animals And the only suggestion we have of any effect of irradiation is that in the irradiated [illegible]

[illegible]

very low incidence of spontaneous thymomas, single large doses of irradiation scarcely increase the incidence, but chronic irradiation [illegible]

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DISCUSSION

Gross: What was the time inter...

... months.

... that you get pituitary tumours following irradiation. Did you ever see pituitary tumours and leukæmia at the same time?

Furth: No. Pituitary tumours appear in mice only among the late survivors (fifteen to thirty months); the incidence increasing with survival time Induced leukæmia occurs earlier. Whether the leukæmic mice had the potentialities to develop pituitary tumours, we do not know

Gross: There is a possibility of ...

... we shall have to pay more a... to the inducing agents in the light of stud... phage We know that ... they get old, for ... injecting urethar... tumours, getting ... time. I ... for this ...

... and ... ther the inducing similar ... latent leukæmic agent, present in many apparently healthy carrier hosts.

350 r is divided into three doses given about a week apart, the leukæmia incidence is raised several-fold, as Kaplan as shown.

LORENZ But it doesn't work in the C3H, you have to fractionate much more

BURCHENAL: Are the irradiation-induced chronic myeloid leukæmias in these mice non-transplantable?

FURTH That has not been studied.

kill any animals acutely, and yet was followed by this very high incidence of a killing tumour. I think possibly it suggests an alternative mechanism to a virus, because in an animal irradiated in that way there is a balance between damage and repair, and repair involves repeated cell division. Sufficient generations of cell division may be all that is required to alter the genes and allow a certain autonomy of growth, an idea that has been suggested before in relation to other sorts of neoplasm.

FURTH: Acute irradiation is certainly very different from chronic. For example, a single dose of about 450 r is but mildly leukæmogenic. If the same dose is fractionated, you may get a much greater or a lesser leukæmogenic effect depending on the interval (Kaplan). I fully agree that repeated exposure is of greater practical hazard, because most people who developed leukæmias did get them following such exposures. What has not been adequately studied are the sensitive and resistant periods, when repeated exposures are given The matter is of the utmost importance to safeguard personnel exposed to intermittent irradiation. Speaking of total dosage of chronic irradiation, without considering the interval biologically, is, I believe, missing a vital aspect of the problem.

FAGRAEUS I am interested in what dose of cortisone was given these mice and for how long?

FURTH 1 mg /day on three consecutive days, this dose brings about complete atrophy of the thymus. In one experiment, this treatment was repeated three times at intervals of seven weeks.

FAGRAEUS. In other work with cortisone it has been found that cortisone had the same effect as X-rays on virus take. For instance, I think Syverton gave half a dose of cortisone and half X-ray, and got the same effect as if he had given a whole dose of either of these. But you find opposite effects—with cortisone an inhibition of leukæmia, and with X-ray an increase in leukæmia?

FURTH Yes, cortisone is not leukæmogenic but is equally capable of lowering the resistance of the host to infections and tumour grafts, presumably by bringing about lymphoid atrophy X-rays are leukæmogenic.

FAGRAEUS What happens if you irradiate a strain with spontaneous leukæmia and a strain that has no leukæmia? From the virus theory you would expect a higher incidence in the leukæmic strain and none at all in the non-leukæmic

FURTH. If you give it to a strain in which the incidence of leukæmia is already high, the effect is likely to be one of reduction of leukæmia incidence, particularly if you extend the irradiation period

FAGRAEUS Is there any strain of mice which is absolutely free of spontaneous leukæmia?

LORENZ C3Hf has an incidence of 0 8 per cent

FAGRAEUS What happens if you give X-ray to them?

LORENZ With acute irradiation, there is only a low incidence of leukæmia, but if we expose the females, beginning at one month of age, to 8 8 r, eight hours per day, then they develop 33 per cent leukæmia.

FURTH: Irradiation doesn't have to be chronic. If, in C57Black mice,

Neither the pre-irradiation measures nor those instituted during irradiation, as described briefly above, have any beneficial effect, as measured by survival, if instituted after the animals have been subjected to whole-body irradiation in the lethal range.

Straube and Patt (1953) have not been able to confirm Bacq's claim (Bacq, *et al.*, 1953) that survival of rats with partial liver-shielding is enhanced when cysteinamine is given after a lethal exposure to whole-body X-radiation

This communication is limited for the most part to a report on measures that, when initiated after exposure of the mammal to whole-body X-radiation in the lethal range, have a remarkable effect on survival and on recovery of the blood-forming tissue A review of our investigations in this field has already been published (Jacobson, 1952) Only a few of these studies are summarized here, more or less chronologically, in an attempt to show how we were led from one step to another, and to emphasize certain interesting but as yet *undeveloped observations.*

These researches were scarcely under way when it became obvious that the success of the various techniques that were employed must be related to one of three mechanisms, namely: (1) the neutralization of a toxin produced by irradiation, (2) the cellular seeding of the depleted irradiated tissue; or (3) a humoral factor or factors produced and supplied by cells to the irradiated tissue. Many of the experiments described below were attempts to provide information on the relative importance of these possibilities. Other experiments were undertaken because of an interest in the basic problem of blood formation without regard to the problem of radiation injury

The Effect of Splenectomy on the Toxicity of ^{89,90}Sr

It was found that mice* given ^{89,90}Sr in a dose of 2 0 $\mu c/g.$ of body weight failed to develop an anæmia even though the

*Mice used in these experiments were CF No 1 females, ten to twelve weeks old, raised by Carworth Farms—homozygous for recessive genes aa, bb, cc.

STUDIES ON THE MODIFICATION OF RADIATION INJURY

L. O. JACOBSON, E. K. MARKS, E. O. GASTON and E. L. SIMMONS

WHOLE-BODY exposure of the mammal to ionizing radiations (X-rays, gamma rays, and fast neutrons) in the midlethal (LD50) or lethal range (LD100) produces a characteristic clinical and pathological picture that has been described in detail by numerous investigators (Minot and Spurling, 1924; Jacobson, Marks and Lorenz, 1949, Lorenz, *et al.*, 1946; Lawrence and Lawrence, 1936, Henshaw, *et al.*, 1947; Zirkle, 1947; Jacobson, Marks, *et al.*, 1954). There has been much interest in the past and there is a determined effort at present to find effective means of decreasing or increasing the radiosensitivity of tissue. Patt (1953) has written a comprehensive review covering the literature on the *in vivo* and *in vitro* biochemical and physiological effects and has summarized current concepts of the mechanism of action of ionizing radiations.

Efforts to alter radiosensitivity of the mammalian organism or a specific tissue of the body are largely centred around the administration of chemical or biological substances, or the initiation of other measures prior to, during, or after irradiation of the organism Œstrogen (Treadwell, *et al.*, 1943), cysteine (Patt, *et al*, 1949), and glutathione (Cronkite, *et al.*, 1951) markedly enhance the survival of rats and mice when given prior to the administration of whole-body X-irradiation in the lethal range Cysteinamine (Bacq *et al.*, 1953, Rugh and Wang, 1953) has an even more strikingly beneficial effect than cysteine

Likewise, survival is enhanced if, during exposure to lethal dosages of X-radiation, animals are subjected to severe oxygen deprivation (Dowdy, *et al.*, 1950) or cyanide intoxication (Bacq, *et al.*, 1950).

hæmatological studies have been made at four exposure levels, namely, 600 r, 900 r, 1025 r, and 1300 r. After exposure to 1025 r or 1300 r, histological and hæmatological recovery is more rapid and complete in spleen-shielded mice than in mice exposed to 600 r without spleen-shielding (Fig. 2). No anæmia and only a transient leucopenia and thrombocytopenia follow an exposure to 1025 r with spleen-shielding, whereas

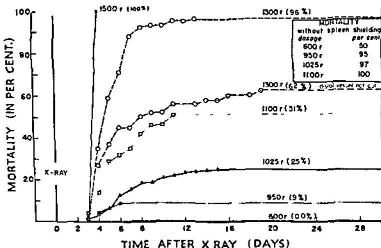


FIG. 1 The effect of spleen-shielding on the mortality of mice following whole-body X-radiation.

pancytopenia and death follow this exposure without spleen-shielding

Histological study of the hæmatopoietic tissues revealed that the characteristic picture of destruction and atrophy follows exposure to 1025 r in both the spleen-shielded and non-spleen-shielded animals; recovery of bone marrow and lymphatic tissue began by four days and was complete by eight days in spleen-shielded mice, but no hæmatopoietic recovery was noted during this interval in non-spleen-shielded mice (Jacobson, Marks *et al.*, 1951). The fact that recovery

bone marrow throughout the body became aplastic within a few days and remained so for a period of more than one month. The lymph nodes became moderately active centres of hæmatopoiesis, whereas the spleen, as early as three days after the injection of $^{89\text{-}90}$Sr, was almost exclusively occupied by erythropoietic tissue. As was expected, a severe and persistent anæmia developed in splenectomized mice that received $^{89\text{-}90}$Sr (Jacobson, Simmons and Block, 1954). Since strontium is physiologically interchangeable with calcium and is almost exclusively a bone-seeker, the lymphatic tissues, including the spleen, were essentially unaffected by its beta radiation and therefore had the capacity to respond to the demand created by the destruction of bone marrow. One of the questions raised by this study was the nature and mechanism of the stimulus to the hæmatopoietic tissue of the spleen. The splenic erythropoietic transformation occurred in the absence of an anæmia, and within three days. Another problem that intrigued us was what would happen if, by some technique, we could destroy all blood-forming tissue in the body except that located in the spleen. This led to the spleen-shielding experiments described below.

The Effect of Lead-shielding of the Spleen on Recovery from Radiation Injury

Lead-shielding of the surgically exteriorized spleen of adult mice during exposure to total-body X-radiation markedly enhances survival (Fig. 1) (Jacobson, Marks, Gaston, Robson and Zirkle, 1949). The survival of mice in which the circulation to the shielded spleen is clamped off during exposure and in which the clamp is released immediately after irradiation is approximately the same as the survival of animals with spleen-shielding without clamping (Jacobson, 1952, Jacobson, Simmons, *et al.*, 1951*a*, 1951*b*) The LD50 for total-body X-radiation is 600 r and the LD99 is around 700 r without spleen-shielding, whereas with spleen-shielding the LD50 is 1100 r and the LD99 is greater than 1800 r (Jacobson, Simmons, *et al.*, 1951*b*). Histological and

Lead-shielding of 0 19 g of kidney does not enhance survival.

These studies raised some interesting speculations. It was apparent that a post-irradiation approach to therapy for radiation injury was possible, since releasing the circulation of the shielded spleen to a mouse that had sustained a lethal exposure to X-radiation was sufficient to ensure survival of the animal.

The shielded spleens of these irradiated animals became the site of intensive blood formation, beginning at the 24-hour interval. By serial sacrifice, it was possible to study the interesting fact that erythropoiesis took precedence over other types of blood formation in the spleen. In fact, in some mice even the lymphatic nodules of the spleen disappeared and only the central artery of the nodule remained at the site of the original nodule. The entire spleen became essentially an exclusively erythropoietic organ and remained so for several days before granulocytopoiesis and megakaryocytopoiesis began to appear Teleologically, one would have expected the reverse to occur, because, theoretically, the animal did not need erythrocytes since no anæmia was present. Leucocytes to combat infection and platelets to ensure capillary integrity and hæmostasis were more likely requirements

The fact that spleen-shielding (0 1 g) was so much more effective in increasing the survival of mice and hastening recovery of the blood-forming tissue than was the shielding of one limb (1 5 g.) or part of the liver (0 8 g), and that kidney-shielding had no effect on survival or blood formation, tended to focus attention on the hæmatopoietic system as the source of whatever was saving the life of the irradiated animals

These observations raised the question of whether survival was increased because the shielded spleen immediately took over blood formation and thus saved the animal from death due to pancytopenia and its complications Consequently, experiments were designed to determine how long the

in the bone marrow by heteroplasia was so prominent was considered to be a particularly significant observation. This will be discussed later.

Shielding of other parts of the body of the mouse, such as a leg, a fraction of the liver, or the intestine, during X-radiation

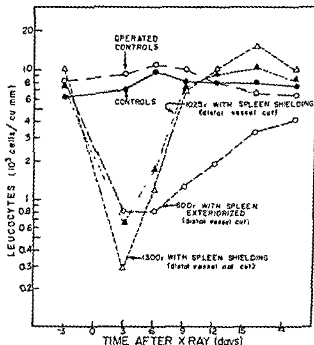


FIG 2 Comparative effect of 600 r whole-body irradiation without spleen-shielding, and 1025 r and 1300 r with spleen-shielding on the leucocytes of mice.

in the lethal range (1025 r), increases survival, but less so than spleen-shielding, even though the volume shielded in each instance is much greater For example, the survival of mice subjected to 1025 r with part of the exteriorized liver (0·8 g.) shielded with lead is approximately 30 per cent compared with 76 per cent survival of mice with spleen-shielding. The average spleen weight in these mice is 0 1 g.

Lead-shielding of 0·19 g of kidney does not enhance survival.

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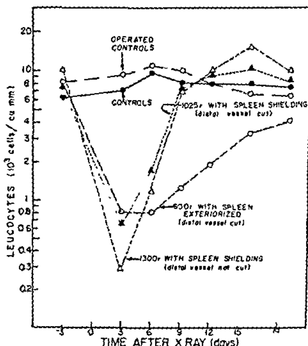


FIG 2. Comparative effect of 600 r whole-body irradiation without spleen-shielding, and 1025 r and 1300 r with spleen-shielding on the leucocytes of mice

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spleen was removed fifteen minutes after the spleen-shielding procedure and was of greater severity and duration than in animals in which the spleen remained in the circulation for one hour.

To exteriorize the spleen for the shielding procedure, it was necessary, for the sake of speed and ease, to sever a small

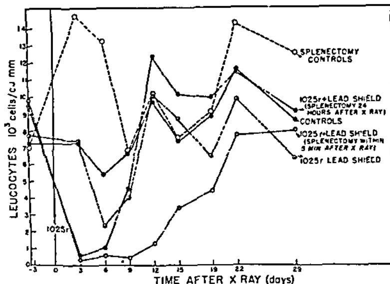


FIG 4. The effect of post-irradiation splenectomy on the leucocytes of mice

vessel at the distal end of the organ, which is not connected with the main splenic pedicle. This left the main pedicle intact, but invariably up to one-half of the distal portion of the spleen was infarcted by this procedure. With this technique, the survival of mice given 1300 r was 3 2 per cent, whereas if this small vessel was left intact and thus no splenic infarction occurred, survival after 1300 r was 31 2 per cent.

It was observed, on the other hand, that during the spleen-shielding procedure the entire splenic circulation was occasionally embarrassed by twisting of the spleen or stretching

originally-shielded spleen had to remain in the circulation of the irradiated animal to ensure survival. This was answered by the series of splenectomy experiments described below.

The Effect of Post-irradiation Splenectomy on Survival of Spleen-shielded Mice

Mice with spleen-shielding were given 1025 r total-body X-radiation. At intervals beginning at fifteen minutes after

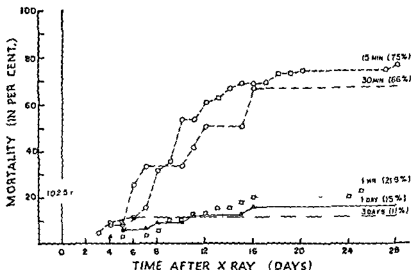


FIG 3 The effect of post-irradiation splenectomy on the mortality of spleen-shielded mice

this procedure, splenectomy was performed (Fig. 3). As short a period as fifteen minutes was sufficient to increase survival. In those animals in which the spleen was left intact for one hour, survival (78 per cent) was comparable with that of the mice given routine spleen-shielding. It was noted that recovery of blood-forming tissue was more rapid and more complete in the animals in which the spleen was left intact in the circulation for the thirty-day period of observation (Fig. 4). A pancytopenia occurred in animals in which the

(25 per cent) even if the animals received the spleens as late as two days after irradiation.

Table I

THE EFFECT OF SPLEEN TRANSPLANTS ON THE SURVIVAL OF IRRADIATED MICE

Number of mice used	*Spleen implants within ½ hour after irradiation*	*Survival (Per cent)*
112	None	0
63	4 spleens from one- to twelve-day-old mice	38
18	2 spleens from four- to five-week-old mice	45
24	4 spleens from one- to eight-day-old mice implanted two days after irradiation of the recipient	20

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It was found that survival was related to the amount of tissue that was implanted. For example, there was 38 per cent survival with 10 mg of baby spleen but none with 5 mg. This was not surprising in view of our previous observation that survival was less in animals in which a partial splenic infarction was produced deliberately than in animals in which no splenic infarction occurred

These observations on survival and others on recovery of blood-forming tissue were interpreted by us to be evidence that a quantitative relationship exists between the amount of tissue furnishing the necessary factor or factors to the irradiated animal and the survival or recovery from irradiation

One experiment involving transplants of baby spleens was especially interesting. Mice were exposed to 1025 r total-body X-radiation and were each given intraperitoneally four baby spleens. Autopsy revealed that in four of twelve mice that survived the twenty-eight-day period of observation there was no trace of the original transplant. In one of the twelve mice only one splenic transplant was found, in three of the mice, two were found; in one, three of the transplanted spleens were found, and in three of the mice, four spleens were found.

of the pedicle. Under these circumstances, even before the spleen was returned to the abdomen its deeply cyanotic appearance indicated that infarction of the entire organ would be likely or had already occurred. Many of these animals lived. By the sacrifice of some of these animals at appropriate time intervals, we proved to our satisfaction that infarction of the entire spleen had occurred and that splenic reconstitution began as the blood supply to the spleen was re-established. The partially reconstituted spleen was capable of initiating the recovery process.

This observation suggested that a splenic transplant should be effective in bringing about the recovery of irradiated animals. If circulation to splenic transplants would become established in the irradiated hosts, then this procedure should increase survival and effect recovery of blood-forming tissue.

The Effect of Splenic Transplants and other Homologous Tissue on Recovery from Radiation Injury

Spleen transplants

Spleens from adult mice were first employed in the transplant procedure and were found to be ineffective. We thus assumed that this failure was due to the fact that when adult spleens were placed randomly in the peritoneal cavity they usually failed to develop a vascular supply. After we found that spleens from baby mice were effective (see below), we discovered that if the capsule of the transplanted adult spleen was cut before it was placed in the peritoneal cavity, it vascularized and significantly enhanced survival. The initial failure with mature spleens prompted a trial with the spleens of baby mice. Spleens (10 to 50 mg) from normal baby mice ranging in age from one to twelve days were transplanted into mice that had been exposed to 1025 r total-body X-radiation. The spleens were implanted intraperitoneally through a small slit in the abdominal wall. No attempt was made to anchor the tissue Thirty-eight per cent of irradiated hosts survived (Table I). Appreciable survival was observed

are less toxic if the cells are removed from the spleen immediately after it is taken from the body of the donor mouse. This may be accomplished by washing the cells from the spleen by multiple injection of saline or Locke's solution, by gently mashing the spleen in a mortar, or by pushing the splenic tissue through a 2-cc syringe. The survival of animals exposed to 900 r and then given an intravenous injection of splenic cell suspensions prepared, as described above, from the spleens of young mice is approximately 75 per cent.

The fact that control transplants, suspensions, or the shielding of such tissues as muscle, kidney, or placenta do not increase survival of the irradiated mouse, whereas transplants or shielding of tissues from major blood-forming tissues such as spleen, liver, or leg were effective, pointed to the so-called reticuloendothelial system as the richest source of the effective substance or substances (Jacobson, 1952) It was logical to try bone marrow transplants and injections even though this technique had previously been tried and reported of no value in dogs (Rekers, *et al.*, 1950, Talbot and Pinson, 1951).

Homologous bone marrow

Lorenz first reported the efficacy of bone marrow injection in increasing the survival of irradiated mice. He found that 75 to 90 per cent of mice given a single exposure to X-radiation in the lethal range (900 r) survived if, after irradiation, a suspension of homologous bone marrow was injected intravenously (Lorenz, *et al.*, 1951). We have corroborated his findings (Jacobson, 1952) and have found that intravenous injection of bone marrow cells also significantly increases the survival of mice exposed to 1025 r. A study of the optimum and minimum number of cells from bone marrow, spleen, or embryo suspensions that will produce significant survival is under way. Preliminary studies appear to indicate that cell suspensions of the soft portions of the embryo are more effective in enhancing the survival of irradiated mice than spleen or bone marrow suspensions. In these studies, each preparation is adjusted so that a comparable number of cells from each

These data suggested that the transplants either vascularized temporarily and were later detached and removed by phagocytosis, or that cells from the splenic transplant migrated out before death and absorption of the transplant had occurred. Whatever the fate of the transplant, it effectively altered the fate of the animal.

Embryo and spleen preparations

The fact that splenic transplants from baby mice increased the survival of irradiated mice and the observation that mice occasionally survived 1025 r even though the transplanted tissue could not be found prompted us to study the effectiveness of embryo and spleen preparations. Mouse embryos (fourteen- to twenty-day) were obtained by cæsarean section. These were ground in saline and administered intraperitoneally to mice exposed to 1025 r. Survival was found to be about 30 per cent (Table II) (Jacobson, 1952).

Table II

THE EFFECT OF EMBRYO SUSPENSIONS ON THE SURVIVAL OF IRRADIATED MICE

Group	*Number of mice*	*X-ray dose*	*Embryo Suspension*	*Per Cent Survival*
Control .	123	1025 r	No	0·8
Treated	123	1025 r	Yes	30 0

Spleens from one- to nine-day old mice were mashed through a tissue press and suspended in buffered saline When the suspension was injected intraperitoneally into mice that had been exposed to 1025 r, there was no survival. In fact, these mice died sooner than was expected from the radiation alone. We found, however, that if the mice were exposed to 800 r, an intraperitoneal injection of this material significantly increased survival (Jacobson, Marks, *et al*, 1951). We later found that smaller amounts of the suspension reduced the toxicity of the preparation and decreased mortality following exposure to 1025 r. We have learned that splenic suspensions

are less toxic if the cells are removed from the spleen immediately after it is taken from the body of the donor mouse. This may be accomplished by washing the cells from the spleen by multiple injection of saline or Locke's solution, by gently mashing the spleen in a mortar, or by pushing the splenic tissue through a 2-cc syringe. The survival of animals exposed to 900 r and then given an intravenous injection of splenic cell suspensions prepared, as described above, from the spleens of young mice is approximately 75 per cent.

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source is injected. The variability in results with various numbers of cells from bone marrow, spleen or embryo suggests the possibility that the type of cells in the injected suspension may be extremely important. For example, the endosteal, reticular, and endothelial cells may be more important than erythroblasts, myelocytes, and megakaryocytes in initiating recovery. We are studying preparations from the various sources to determine actual cellular make-up and the proportions of the constituent cells in an attempt to relate cell type to survival data.

Heterologous tissue

Transplantation of baby mouse spleens into the peritoneal cavity of irradiated rabbits gave evidence that suggested that the regeneration of the rabbit hæmatopoietic tissue was hastened by this technique (Jacobson, 1952) It has more recently been demonstrated by Lorenz and Congdon that the intravenous administration of guinea pig bone marrow (Lorenz, Congdon and Uphoff, 1952) or rat bone marrow (Congdon and Lorenz, 1954) will markedly increase the survival of mice that have been exposed to X-radiation in the lethal range. We have corroborated his findings on the effectiveness of rat marrow in mice (Jacobson, 1954).

As suggested in a previous paragraph, the fate of individual injected cells is not known with any degree of certainty. Whether suspensions of heterologous and homologous bone marrow or (washed) spleen cells implant in the body and live a "normal life span", or whether these cells only temporarily function as normal cells is unknown. For example, it is not known whether rat hæmatopoietic cells injected into an irradiated mouse function in phagocytosis and divide and multiply. It would appear that by some mechanism these cells succeed in altering the pathological state of the irradiated animal to the extent that the animal recovers.

It is assumed by many, and perhaps rightfully, that it is recovery of the blood-forming tissue that initiates or fosters the recovery process in irradiated animals treated by one of

the various techniques under discussion. It is obvious that recovery of bone marrow and lymphatic tissue is initiated under these circumstances by four days after irradiation (and perhaps it would be found to be earlier if methods of studying functional reconstitution of cells were available) That the normal function of these tissues is vital in combating infection has been shown by Miller *et al.* (1952). By way of speculation, however, one might logically raise the question of why the overwhelming infection, which is described by these authors, comes relatively late after lethal amounts of irradiation even though destruction of the blood-forming tissue is manifest soon after the exposure. Is there a mechanism apart from the blood-forming tissue that protects the animal against bacterial invasion during the first week after irradiation?

One should not assume that because blood-forming tissue does recover it is the only one vital to survival. Other tissues such as the liver may be quite important. Perhaps we only lack methods sensitive enough to study the *in vivo* inhibition and recovery process of these tissues after irradiation There is good evidence that recovery of blood-forming tissue is not the only factor essential to recovery One has only to consider the data referred to above, namely, that only *circa* 30 per cent of animals survive 1300 r with spleen-shielding and preservation of the entire splenic circulation, whereas 3 2 per cent survive if the spleen is shielded and only part of the splenic pedicle is preserved In the former group one might expect nearly 100 per cent survival if recovery of blood-forming tissue were the only factor involved Williams and DeLong (1953) have reported experiments in which the entire body of the rat was shielded except for an exteriorized segment of the small bowel. The rate of recovery of the bowel epithelium in this segment was not appreciably different from that observed in control non-shielded animals Their work implies that recovery of bowel epithelium or failure to recover from radiation injury is largely, if not entirely, unrelated to the state of the blood-forming tissue These observations emphasize the limitation of the "therapeutic" techniques described

in this communication and focus attention on the importance of systems other than the blood-forming tissue that are vital to recovery from radiation injury. The radiosensitivity of cells in various tissues in the body such as the brain, thyroid, and gonads varies widely. Cells derived from a single source, such as the reticulum of the blood-forming tissue, have different radiosensitivities (Bloom and Jacobson, 1948). There is evidence to show that various functions of the same cell have different radiosensitivities. For example, reticular cells are capable of phagocytosis. This function is extremely radioresistant, and exposures above 1000 r seem not to affect it (histological observation), whereas heteroplastic or homoplastic multiplication is inhibited by about 500 r. It may also be assumed that with increasing exposures not only are more and more functions of the reticulum eliminated, but other cells in the body are similarly affected.

Synergistic Action of "Prophylactic" and Therapeutic Measures on Radiation Injury

It has been shown, as mentioned in the introduction, that compounds such as œstradiol and cysteine, when given prior to irradiation, enhance the survival of irradiated animals. Such prophylactic measures combined with post-irradiation measures such as spleen-shielding have an additive effect on survival (Bethard, *et al.*, 1951, Simmons, *et al.*, 1951). These findings do not necessarily imply that the mechanism of action of both procedures is identical.

Attempts to Extract the Factor or Factors from Hæmatopoietic Tissue

We have made a number of attempts to obtain a cell-free extract of spleen, embryo, and bone marrow that would increase the survival of irradiated mice Thus far our results have been negative or equivocal. For example, simple buffered saline extracts of spleen, bone marrow, or embryo, or homogenized preparations of such tissue, produce negative results if the material is filtered and thus no cells or parts of cells are

present. On the other hand, we have centrifuged cell suspensions of spleen and bone marrow and have obtained positive activity in the supernatant. However, we have never been able to eliminate cells in the supernatant by centrifugation.

Cole and his co-workers (1952, 1953) claim to have prepared suspensions of mouse spleen cells with only the nuclei of the cells intact. According to their report, such preparations, when administered intraperitoneally to mice that have been given a lethal exposure to X-radiation, will routinely increase survival. If, indeed, these suspensions contain no intact cells and thus no living cells in the ordinary sense, then additional proof for the humoral theory is at hand. Since, however, identification of the effective substance or substances in the whole cell or the nucleus still remains to be solved, the need for a simple and sensitive method to assay the activity is obvious. Survival following lethal irradiation is currently being employed to determine the effectiveness of various cells suspensions or preparations free from living cells. This method is probably too insensitive for the problem

Relation of Species and Strain to the Effectiveness of Spleen-shielding or Injection of Hæmatopoietic Cell Suspensions

The phenomenon of hastened hæmatopoietic recovery as well as increased survival in animals X-radiated in the midlethal or lethal range with spleen-shielding is not confined to the mouse. Lead-shielding of the spleen of rats, guinea pigs, and rabbits during midlethal exposures is followed by a more rapid recovery of the hæmatopoietic tissue and a reduced mortality. However, there is a pronounced difference in the species response to the techniques under discussion. For example, spleen-shielding in the mouse spectacularly increases survival of the animal and markedly hastens the recovery of blood-forming tissue, whereas a definite but minimal effect is observed in the rabbit. This may be due to a quantitative difference in the capacity of the rabbit spleen or appendix

to produce the substance or substances necessary for the recovery process as compared with the mouse.

There are also strain differences. We have studied six different strains of mice and have found that spleen-shielding or injection of suspensions of hæmatopoietic cells after total-body X-radiation effectively increases survival of all of the strains and hastens hæmatopoietic regeneration. In addition, bone marrow, spleen or embryo suspensions from one mouse strain are effective in another strain. However, in one particular strain (CF No. 1) the LD50 with spleen-shielding is about 1100 r whereas in another mouse strain (Webster) the LD50 with spleen-shielding is 1000 r.

Effect of Spleen-shielding on Antibody Formation

It is well known that total-body X-radiation inhibits antibody formation in laboratory animals (Hektoen, 1915, 1918). The capacity of rabbits exposed to 800 r total-body X-radiation with spleen- or appendix-shielding to produce antibodies in response to an intravenously administered particulate antigen is retained, whereas in irradiated rabbits without shielding this capacity is suppressed (Jacobson, *et al.*, 1950). In fact, the capacity to produce antibodies to an injected antigen is retained in irradiated rabbits even though the originally-shielded spleen is removed twenty-four hours after the shielding-irradiation procedure and the antigen is given forty-eight hours after the exposure (twenty-four hours after splenectomy) (Jacobson and Robson, 1952). In the experiments referred to above, the antigen was administered (both in the experimental and control animals) when the cellular hæmatological constituents of the peripheral blood were greatly reduced, cell death and phagocytosis of hæmatopoietic tissue were continuing, and there was no evidence of regeneration. Nevertheless, antibody formation was initiated [illegible] circulation of the rabbit for three hours after total-body

X-radiation to restore significantly the capacity to produce antibodies (Jacobson and Robson, unpublished observations).

Discussion of Theories on Mechanism of the Effect of Spleen-shielding and Related Techniques on Recovery from Radiation Injury

The theories on mechanism of these techniques that have been obvious to us since the original observations are three:—

1. Detoxification of substances produced by irradiation.
2. Cellular seeding and tissue reconstitution.
3. Production of a humoral substance that initiates functional reconstitution of cells

It has long been assumed that irradiation results in the production of a toxin that exerts an inhibitory effect on metabolic processes within the irradiated cells. Transport of this "toxin" to other parts of the body from the irradiated site also inhibits presumably cellular processes at distant non-irradiated sites. The production of hydrogen peroxide along the path of ionization has been suggested (Barron, *et al.*, 1949). It is conceivable that the local and distant effect may be the result of the action of this or some other oxidizing substance. However, if this is true, one must assume that the production and dissemination of the substance and the initiation of the biological effects occur within a relatively short time after irradiation and that the "toxin" is then destroyed or neutralized. Evidence for this is briefly as follows. After the administration of lethal amounts of radiation to groups of mice with or without spleen-shielding, the initial pathological effects are identical. For example, destruction and atrophy of the lymphatic tissue and bone marrow proceeds in both experimental groups at the same rate and reaches the same maximum. The observable difference in the two groups is that recovery of the blood-forming tissue begins by the fourth day in the spleen-shielded group and about 75 per cent of animals survive, whereas there is little or no histological evidence of recovery of the blood-forming tissue in the

non-spleen-shielded group and death is inevitable. If one removes the spleens from these two experimental groups one hour after irradiation, survival and recovery of hæmatopoietic tissue is nil in the non-spleen-shielded group, whereas survival is 75 per cent and recovery of hæmatopoietic tissue begins at four days in the spleen-shielded animals. This may be evidence that if a toxin is indeed responsible for the destruction and continued inhibition of cells, it must be a transitory one or it would continue to act beyond the one hour during which the spleen is left intact in the circulation in the spleen-shielded group, and these animals would not be expected to recover. It seems unlikely but it cannot be denied, however, that while the spleen is in the circulation it may continue to neutralize the toxin and that after removal of the spleen, cells that came from the spleen and were disseminated throughout the body carry on the detoxification process. This aspect will be discussed more fully.

Another interesting observation that might be used as evidence against the continuing production and dissemination of a toxin is that the injection of spleen cells or bone marrow is effective in reversing the otherwise lethal effect of radiation even when either is administered two or three days after the recipient animal has been exposed to a lethal amount of radiation (Jacobson, Marks and Gaston, unpublished observations) If a specific toxin were entirely responsible for the destruction and continued atrophy and inhibition of cells and organ systems, one might logically expect an increasing pathological state. It has been suggested by Storer *et al* (1952) that the effectiveness of shielding is related to the capacity of the shielded tissues to forestall infection until the tissues of the body and especially the blood-forming tissue can regenerate spontaneously This seems an untenable explanation for a number of reasons, one of which is the fact that the shielded spleen of the mouse can be removed an hour or less after the administration of a uniformly lethal total-body exposure and yet the animals survive The effectiveness of heterologous cells (rat to mouse, guinea pig to mouse, and

mouse to rabbit) to bring about recovery, makes Storer's postulate even more untenable unless the heterologous cell potential has not yet been elucidated. It is hard to believe that rat bone marrow cells injected into a mouse an hour after it has been irradiated will live for a number of days and control infection.

The theory that shielded tissue and injected hæmatopoietic cell suspensions are effective in reversing the lethal effects of radiation by seeding destroyed hæmatopoietic tissue throughout the body is attractive but seems unlikely. The effectiveness of heterologous tissue makes this theory rather remote. One cannot ignore the histological observation that in spleen-shielded mice or mice injected with cell suspensions, hæmatopoietic regeneration in the bone marrow from reticular cells and osteoblasts is prominent. If seeding via the blood stream and subsequent regeneration by multiplication of cells is a factor in the regeneration, then the release of the inhibition of the reticular cells, allowing them to carry on multiplication, would still have to be explained

It would appear more reasonable that the effect produced by lethal irradiation represents a chain of events initiated by an agent such as hydrogen peroxide, and that the profound effect of spleen-shielding, or injection of such materials as embryonic tissue, spleen or bone marrow cells and the like could best be explained by assuming that these cells or tissues provide one or more metabolites necessary for functional reconstitution of the injured cells Cole's observations, referred to previously, and the effectiveness of heterologous tissue, materially strengthen this hypothesis.

Summary

Whole-body exposure to ionizing radiations in the lethal or midlethal range produces widespread destruction of certain tissues in the body such as blood-forming tissue, the gastrointestinal tract, and the gonads. Death may be related to injury of one or more tissues and organ systems, but the destruction and delayed recovery of blood-forming tissue is

considered to be one of the more critical factors. A number of prophylactic measures such as the injection of cysteine and glutathione just before irradiation will reduce mortality. These materials have no beneficial effect if given afterward. Measures that in effect are instituted after lethal irradiation, such as spleen-shielding or the injection of spleen cells, suspensions of spleen or embryo cells, and bone marrow, increase survival and hasten recovery of blood-forming tissue. The mechanism of this striking effect is unknown. The possibilities considered are: 1. detoxification of some radiation-produced toxin; 2. seeding of the animal with injected cells that multiply and repopulate tissue such as the bone marrow; and 3. production of a humoral substance (or substances) by the shielded or injected cells that brings about functional reconstitution of the inhibited cells and initiates regrowth of vital tissues. On the basis of a number of observations, including the pattern of histological recovery in spleen-shielded animals and the fact that heterologous tissue (e.g. rat to mouse) effectively enhances the survival of irradiated mice, the humoral theory seems the most acceptable.

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DISCUSSION

TURNER. One point which interested me is the extraordinary rapidity with which anæmia develops in these animals. I suppose that could be due to a very short life span where there is an aplastic marrow, but, even so, it is hard to account for the recovery with the transplanted spleen unless some humoral or detoxification mechanism is involved.

JACOBSON. After the exposure of any of the common laboratory animals to an LD_{50} of X-radiation, a reduction of the circulating erythrocytes occurs (reduction of about 30 per cent), reaching a minimum by about fourteen days. Thereafter recovery ensues If the dose is raised to 1000 r (in mice), the drop is precipitous: a reduction of 75 per cent in less than sixty days. This cannot be accounted for by aplasia of the bone marrow alone, and thus one must assume that red cells are being destroyed by one mechanism or another If mice are exposed to 1000 r with spleen-shielding or are given bone marrow or spleen cells intravenously after irradiation, this precipitous fall in erythrocytes does not occur ...
thi
of
is
Dr. Furth has done much work in this field and can perhaps clarify the problem

FURTH The pathogenesis of post-irradiation anæmia is complex. After massive irradiation, erythrogenesis ceases almost immediately, but this can account for a loss of only about 1 per cent per day. Massive irradiation is followed by the appearance of erythrocytes in large numbers in the lymph The peak of this erythrocyte diversion into the lymph is reached in rats at nine to fourteen days and in dogs at eleven to seventeen days after irradiation, at which time the erythrocyte counts in the lymph often exceed one million per mm³. There is a relationship between irradiation thrombocytopenia and bleeding into the tissues

... ts have been
... ytes into the
... Our studies
... tain vascular

FURTH I can conceive "toxins" from cell destruction and bacteria to

able, I believe that yours, trying to find and characterize protective substances, is a most fertile area of research

FURTH We did not study survival times Cronkite and Brecher, working with dogs, state that they did save the animals. The anæmic

Furthermore there are other causes of anæmia, e g bacterial hæmolysins and direct injury to vessels, the latter probably occur with doses fatal within a few days

PATERSON I have made graphs of the red cell

red cell count for the surviving monkeys is reduced in a slope which is not unlike the natural length of life of blood corpuscles if one assumed

that they are stopped at the source, it then recovers. But the dying monkeys have a precipitous fall. I think this would tend to indicate that the theory that they leave the blood circulation and go elsewhere is very feasible. In the surviving monkeys the reticulocytes show an enormously high peak at twenty-six days, and in the dying monkeys there is no response whatsoever. That is why I think that the platelet count and the anæmia are not parallel phenomena.

DANIELLI. I showed about 1940 that blood platelets make a vast difference to the permeability of capillaries to cells and also to some extent to proteins, and I think therefore that what Dr. Furth said is supported by some physiological evidence in addition to what he mentioned The technique of these platelet counts is probably totally inadequate in this connection, because you may include both good and bad platelets. We need to know a lot more about platelets before we can judge that. It is difficult to observe if the intracellular cement is intact, but if it has suffered as the result of some treatment, then the effect of the platelets will be much more pronounced.

Another point which might be of interest to Dr. Jacobson is the work of J G Stephens (*J. Physiol.*, 92, 39; 95, 02.) in which he obtained evidence that the spleen can carry out renovations on red cells

JACOBSON. I don't doubt that platelets are important in recovery from radiation injury However, if one exposes mice to 1000 r. and even if one shields the spleen, or gives bone marrow
venously, all the cellular
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I think it is technical

MOTSCHLIN I should if you can produce
the

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grow. But if you freeze and thaw, freeze and thaw, it will not work, because then, presumably, all activity has been lost.

MOESCHLIN. If you protect the spleen and remove it after one or two days (if I understood you properly) you still get a good antibody production Now if you protect the spleen and take it out immediately after the X-ray, do you still get good antibody production?

JACOBSON: In the experiments which I have discussed, the spleen

tumour itself that is doing the job, because even in a tumour one has normal tissue

LORENZ. We took not the original transplant but the metastases in the liver, and we have shown previously that suspension of adult liver does not protect

BURCHENAL What happens to this reticulum cell sarcoma?

LORENZ It kills the mice, in about thirty to forty days

BURCHENAL Has it been tried in guinea-pigs or in rats, or some species where it wouldn't take?

LORENZ It is a very slow-growing tumour, and sometimes there are no liver metastases The tumour itself contains a lot of crystalline material—nobody knows what this is We are trying to get enough

ulation theory
and obviously

FAGRAEUS I think you might be able to rule out the repopulation

FURTH: I should like to mention an interesting experiment of Lushbaugh, who has shown that the yellow bone marrow of the tail of the normal mouse is not protective but can be made hæmopoietic and protective by keeping the tail at body temperature.

LORENZ: We use the long bones of the mouse, that is, bone that normally contains bone marrow.

different radiosensitivities. For example, the capacity to produce antibodies is inhibited by about 250 r, the ability to phagocytize material is not affected by 1000 r; division and multiplication of these cells are inhibited by about 500 to 800 r. This implies that these functions of the same cell require different substances, yet the capacity of these cells to function normally is reinstituted by the techniques under discussion. This may mean that we are dealing with several humoral factors rather than one.

ENGELBRETH-HOLM: You mentioned three possible explanations, and

JACOBSON: That would be detoxification

of
ter
ia.
of

the animals by giving streptomycin, however, all will eventually die. Then you find a still depleted bone marrow. So it is not a detoxification phenomenon.

TURNER: I wondered if one might not take the view that it is a combination of the detoxification and humoral factor theories, something like tuberculin sensitivity transfer.

LORENZ: You can see that for this reason the homologous bone



FIG 1 (Lorenz) Intraperitoneally transplanted guinea-pig bone marrow, twenty days after irradiation

[To face page 188

[illegible]

FURTH: I should like to mention an interesting experiment of Lushbaugh, who has shown that the yellow bone marrow of the tail of the normal mouse is not protective but can be made hæmopoietic and protective by keeping the tail at body temperature.

LORENZ: We use the long bones of the mouse; that is, bone that normally contains bone marrow.

JACOBSON: I was talking to Dr. Thorell last night about a matter [illegible]

[illegible] bodies is inhibited by about 250 r, the ability to phagocytize material is not affected by 1000 r; division and multiplication of these cells are inhibited by about 500 to 800 r. This implies that these functions of the same cell require different substances [illegible]

[illegible] it be the other way around, that something was taken up by the [illegible]

[illegible] toxic.

[illegible] Mice of the C3H strain develop bacteræmia beginning the third day after irradiation. With intravenous bone marrow they get no bacteræmia. So you could say that is detoxification. You can also prolong the life of the animals by giving streptomycin, however, all will eventually die. Then you find a still depleted bone marrow. So it is not a detoxification phenomenon.

TURNER: I wondered if one might not take the view that it is a combination of the detoxification and humoral factor theories, something like tuberculin sensitivity transfer.

LORENZ: You can see that for this reason the homologous bone marrow is always much more effective than the heterologous. We get [illegible]

[illegible] transplanted bone marrow in an inbred strain of guinea-pig looks like twenty days after irradiation with a lethal dose. In a normal guinea-pig, you don't find it, you find more bone and very little bone marrow.

THE RÔLE OF BONE MARROW AND SPLEEN IN INDUCED AND SPONTANEOUS LYMPHATIC LEUKÆMIA

EGON LORENZ, L. W. LAW and CHARLES C. CONGDON

KAPLAN and Brown (1951) have shown recently that total body irradiation will induce lymphoid tumours in C57BL mice, while protection of either the upper or lower half of the body and even the shielding of one thigh will effectively prevent the induction of lymphoid neoplasms. Observation of the modification of irradiation injury by spleen protection during irradiation (Jacobson *et al.*, 1949) or by bone marrow injection, post-irradiation (Lorenz, Congdon and Uphoff, 1952), suggested that prolonged depression of hæmatopoietic tissues may play an important rôle in the induction of lymphoid tumours. Both spleen protection and bone marrow injection result in a rapid recovery of hæmatopoietic tissues of mice given a total body exposure that would otherwise be lethal. In the experiments of Kaplan and Brown, where the thigh was shielded, bone marrow was protected This shielded bone marrow should have an effect similar to that of spleen protection or of the injection of bone marrow. To test the rôle bone marrow plays in irradiation-induced lymphoid tumours, the following experiments were performed (Lorenz, Congdon and Uphoff, 1953). One group of C57BL mice received four total body doses of 225 r once per week; during the first irradiation the spleen was exteriorized surgically but not shielded. The second group received the same irradiation treatment, but during each irradiation the spleen was exteriorized and shielded. The third group was exposed to a single total body dose of 900 r with the spleen shielded The surviving animals were killed and autopsied three hundred

Table I

INCIDENCE OF LEUKÆMIA IN $C3H_f \times AKR\ F_1$ MICE FOLLOWING VARIOUS TREATMENTS

Incidence expressed as $\frac{\text{animals autopsied with lymphoid tumours}}{\text{animals autopsied without lymphoid tumours}}$

Group	*Strain*	*No mice*	*Treatment*	*Age in 30 day intervals*												*Incidence at 14 mos*	*Mean tumour age at 14 mos.*
				3	4	5	6	7	8	9	10	11	12	13	14		
Control	AKR	119	None	—	—	1/0	8/0	10/2	32/4	26/2	11/1	8/1	6/0	1/0	3/0	89%	230 days
Control	$C3H_f$	152	None	—	—	—	—	—	—	—	—	—	—	0/2	0/3	0%	None
Control	$C3H_f \times AKRF_1$	170	None	—	—	—	—	—	—	11/1	7/2	8/1	10/2	11/1	8/3	82%	348 days
I	*$C3H_f \times AKRF_1$*	44	AKR B M * I V	—	—	—	—	2/0	9/0	3/0	2/0	3/0	1/0	1/0	4/0	55%	281 days
II	$C3H_f \times AKRF_1$	48	$C3H_f$ B M I V	—	—	—	—	—	—	—	—	1/0	1/0	0/1	4/0	13%	383 days
																Incidence at 11 mos	*Mean tumour age at 11 mos*
Control	$C3H_f \times AKRF_1$	170	None	—	—	—	—	—	—	—	11/1	7/2	—	—	—	11%	297 days
III	$C3H_f \times AKRF_1$	47	X radiation 4 × 225 r	—	—	—	0/1	1/0	4/0	6/0	5/1	1/0	—	—	—	40%	253 days
IV	$C3H_f \times AKRF_1$	46	X-radiation 4 × 225 r + 4 × AKR B M I P	—	1/1	14/0	2/0	2/0	5/0	2/0	7/0	2/0	—	—	—	76%	200 days
V	$C3H_f \times AKRF_1$	47	X radiation 4 × 225 r + 4 × $C3H_f$ B M I P	—	—	—	—	—	3/2	6/1	2/0	8/0	—	—	—	40%	275 days

*Intact bone marrow cells (B M) given intravenously (I V) or intraperitoneally (I P.)

days after the first irradiation, since at that time the incidence of lymphoid tumours reaches a plateau.

The results are as follows: In the first group without spleen protection, a tumour incidence of 68 per cent was observed. In the second spleen-protected group, the tumour incidence was 2 per cent, and in the third spleen-protected group, exposed to a single total body exposure of 900 r, no tumours were found. It is evident from these data that spleen protection effectively inhibits the induction of lymphoid tumours in C57BL mice. While the number of circulating lymphocytes showed a depression of long duration in the non-spleen-protected group, this depression was less pronounced and of shorter duration in the spleen-protected groups. Concomitantly, the bone marrow depression observed in the first group at the termination of the experiments was absent in the second and third group. These observations indicate that prolonged bone marrow depression accompanied by prolonged reduction of lymphocytes in lymphoid structures may be a major factor in the irradiation induction of lymphoid tumours in mice.

These findings suggest that bone marrow may also play a rôle in spontaneous mouse leukæmia. To test this possibility a series of experiments were undertaken using F_1 hybrid mice derived from crosses of low-leukæmic mothers, the $C3H_f$ strain, and high-leukæmic fathers, the AKR strain. The incidence of leukæmia in the F_1 test mice is approximately 50 per cent, the incidence of the father strain being close to 100 per cent and that of the mother strain being approximately 1 per cent during their life span. F_1 hybrids were chosen because it is to be expected that bone marrow of the father strain as well as that of the mother strain will stay alive in the hybrid At the age of three months, when the hybrids are still non-leukæmic, one group was injected intravenously with the bone marrow of one- to three-months-old mice of the high-leukæmic father strain (AKR) which at that age is also still non-leukæmic (see Table). The second group received bone marrow intravenously from one-month-old

hybrids as well as in the leukæmic donor strain. Tumours arising in the hybrids following injection of low-leukæmic bone marrow, when transplanted, grew only in the hybrids. This indicates that the bone marrow of the high-leukæmic strain contains already, at one to three months of age, cells which have the potentialities to develop into leukæmia, although frank leukæmia is usually not observed until five months of age. On the other hand, a single injection of low-leukæmic bone marrow appears not only to decrease the incidence of leukæmia but also to increase the age at which leukæmia appears. Full evaluation of these findings, however, cannot be made until the data for the full life span of the animals are available.

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DISCUSSION

MOESCHLIN I think it is very interesting that Dr Lorenz could transfer leukæmia by bone marrow transplants to the other animals before leukæmia was manifest It has long been believed that leukæmia in humans starts a long time before it is clinically apparent. We think that in chronic myeloid leukæmia two to three years may pass before

her eosinophils rose to about 8–10 per cent, but not her leucocytes. In the twelfth year her leucocytes started to rise, and in the sixteenth year she died in a typical chronic myeloid leukæmia with terminal myeloblast reaction So in that case one really could follow the whole evolution of

reciprocal crosses?
Dr Gross has not as
Suppose the virus

mice of the mother strain ($C3H_f$). The third, fourth and fifth group received four doses of 225 r total body irradiation once per week. Groups IV and V received in addition, following each irradiation, bone marrow from one-month-old mice of the high-leukæmic strain or the low-leukæmic strain respectively. The first injection was made intravenously, the other three intraperitoneally. In the upper part of the Table, data on tumour incidence are given for the non-irradiated bone marrow injected mice to an age of fourteen months, together with data on normal tumour incidence in the AKR and $C3H_f$ strains and the F_1 hybrids; in the lower part data on irradiated and irradiated and bone marrow injected mice to an age of eleven months. For comparison the incidence of leukæmia in untreated hybrids is given for the same age.

Although the experiments are not complete since not all the mice have died, certain conclusions can be drawn from the data obtained to date. When the hybrids were injected with bone marrow of one- to three-months-old mice of the high-leukæmic father (AKR) strain (Group I) the incidence of leukæmia in the hybrids increased from 32 per cent to 55 per cent On the other hand, the incidence of leukæmia decreased to 13 per cent (Group II) when bone marrow of the low-leukæmic mother strain was injected.

X-irradiation alone increased the incidence of leukæmia at eleven months of age to 40 per cent (Group III) in comparison to 11 per cent in untreated hybrids at eleven months. Injection of bone marrow of the high-leukæmic father strain (AKR) following irradiation (Group IV) raised the incidence to 76 per cent, while injection of low-leukæmic bone marrow ($C3H_f$) following irradiation (Group V) had no influence upon the incidence of leukæmia. This is not surprising in view of recent findings of Kaplan (Kaplan, Brown and Paull, 1953) that intraperitoneal bone marrow injections failed to influence the incidence of irradiation-induced leukæmia in C57BL mice.

Transplantation experiments showed that leukæmia arising in the hybrid animals which had received bone marrow from one-month-old mice of the high-leukæmic strain grew in the

hybrids as well as in the leukæmic donor strain. Tumours arising in the hybrids following injection of low-leukæmic bone marrow, when transplanted, grew only in the hybrids. This indicates that the bone marrow of the high-leukæmic strain contains already, at one to three months of age, cells which have the potentialities to develop into leukæmia, although frank leukæmia is usually not observed until five months of age. On the other hand, a single injection of low-leukæmic bone marrow appears not only to decrease the incidence of leukæmia but also to increase the age at which leukæmia appears. Full evaluation of these findings, however, cannot be made until the data for the full life span of the animals are available.

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DISCUSSION

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reciprocal crosses?
Dr Gross has not as
Suppose the virus

is in the AKR stock, and doesn't go through the male, and perhaps these F_1's don't have any virus in them. You have found that the incidence is 40 per cent if they are irradiated only, 40 per cent if they are just given C3H marrow, but 76 per cent if they are given AKR marrow.

ciprocal crosses.

We have done some other work on transplantation of AKR thymic transplants to these hybrids. In each case the thymic fragment became leukæmic after a period of time, and upon transplantation these thymic fragments behaved like F_1 leukæmias and not like AKR leukæmias, which were shown here in two out of five cases

DUSTIN: I am interested in the relation between the depression of lymphocyte formation and the incidence of leukæmia. Cortisone produces a similar depression of lymphocytes Does prolonged administration of cortisone increase the percentage of lymphoid leukæmia?

Tl

Cc

irradiation also affects the hæmopoietic reticulum cells

DUSTIN: But wouldn't you say that irradiation produces genetic

ISRAËLS: Have you tried transplanting the thymuses of the ones you protected?

LAW: I haven't done any of that work.

FURTH: Depression of resistance by irradiation is commonly attributed to lymphocyte depression. If this is true, lymphocytes infused in large numbers may be protective. With this in mind, associates of

mine tapped the thoracic or mesenteric duct of rats with plastic cannulæ, observed and counted the lymphocytes with the phase-contrast microscope and injected enough to bring the lymphocyte level of irradiated animals back to normal, but there was no protection. So we eliminated the lymphocyte as the protecting cell, perhaps too hastily. I now suspect that the hæmopoietic reticulum cell might afford protection.

With regard to the last part of Dr. Lorenz's discussion—namely, the latency of neoplastic cells—this is a remarkable phenomenon which has been amply documented. In irradiated tissues, one can see cells which morphologists would consider malignant—cells with giant nuclei and chromosome abnormalities; yet, they remain localized Dr Rous and others have shown that there are several phases in carcinogenesis, one with cells already altered but remaining latent until a second stimulus which can be non-carcinogenic such as croton oil, will bring about tumour growth. Dependent pituitary tumour cells can remain latent in normal hosts during about one-third of the life span of the animal. So, by inference, we should also think of phases in leukæmogenesis.

EXPERIMENTAL AND CLINICAL ASPECTS OF THE ACTION OF VARIOUS CARBOXYLIC ACID DERIVATIVES IN THE AROMATIC NITROGEN MUSTARD SERIES

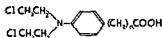
A. HADDOW

THE work to be presented in this communication is a recent outcome of a large-scale investigation started six years ago in collaboration with the late Professor G. A. R. Kon, Dr. W. C. J. Ross and others, (Haddow, Kon and Ross, 1948), the purposes of which were: firstly, to endeavour to improve the therapeutic efficacy of the nitrogen mustards by study of a large number of derivatives in the aromatic series; and secondly to elucidate their fundamental mode of action. The biological test object initially used was the Walker rat carcinoma 256, subsequent tests being extended, as indicated, to include other tumours of the rat and mouse, spontaneous, transplanted and induced. this procedure had meanwhile been successful in detecting the inhibitory effects of such agents as the synthetic œstrogens, urethane, and "Myleran" (1 : 4-dimethanesulphonyloxybutane, Haddow and Timmis, 1953). At a fairly early stage there emerged a certain association between biological action and chemical reactivity: this is illustrated in Table I, and proved an important factor in directing the course of the work in its fundamental aspects.

The entire series now represents a large number of types of chemical modification, including the sub-series to which the present communication refers, namely the carboxylic acid derivatives shown in I, which are due in the main to Dr. W. C. J. Ross. Of these compounds, the member in which n=3 (CB1348=*NN*-di(2-chloroethyl)-*p*-aminophenylbutyric acid) proved of special interest in so far as it is the first substance, at least in the writer's experience, capable of producing, when

Table I

COMPOUND	% HYDROLYSIS IN ½ HR IN 50% ACETONE AT 66° (ROSS)	BIOLOGICAL ACTIVITY (HADDOW)
o-$CH_3OC_6H_4N(CH_2CH_2Cl)_2$	89	+
o-$CH_3C_6H_4N(CH_2CH_2Cl)_2$	83	+
p-$CH_3OC_6H_4N(CH_2CH_2Cl)_2$	58	±
p-$NHAcC_6H_4N(CH_2CH_2Cl)_2$	41	+
p-$CH_3C_6H_4N(CH_2CH_2Cl)_2$	38	+
m-$CH_3C_6H_4N(CH_2CH_2Cl)_2$	21	+
$C_6H_5N(CH_2CH_2Cl)_2$	20	+
$C_6H_5C_6H_4N(CH_2CH_2Cl)_2$	12	−
p-$ClC_6H_4N(CH_2CH_2Cl)_2$	9	−
p-$CO_2EtC_6H_4N(CH_2CH_2Cl)_2$	1	−
p-$CHOC_6H_4N(CH_2CH_2Cl)_2$	<1	−
$2,4$-$(NO_2)_2C_6H_3N(CH_2CH_2Cl)_2$	<1	−



I

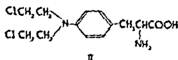
administered shortly after implantation, complete inhibition of the growth of the Walker tumour. This effect is illustrated in Table II and, although striking, is most probably due not

Table II

ACTION OF *NN-DI*(2-CHLOROETHYL)-*p*-AMINOPHENYLBUTYRIC ACID (2 MG ADMINISTERED DAILY BY FEEDING) ON THE GROWTH OF THE WALKER RAT CARCINOMA 256. TUMOUR WEIGHTS (G.) AT FOURTEEN DAYS

Control .	35	33	19	16	13	13	9	6
Treated . .	0 0	0 0	0	0	0	0	0	0

to any specific effect of the acid side-chain, but rather to favourable pharmacological properties concerning water- and fat-solubility. When tested against other tumours such as the Crocker sarcoma 180, the sarcoma S-37, the Krebs ascitic tumour and transplantable leukæmias in the mouse, various degrees of inhibition are observed, although in no case are they as striking. The series is still under examination, and an interesting recent development, due to Professor F. Bergel and Dr. J. A. Stock, is the detection of high activity in the "mustard" derivative based upon phenylalanine (II)



This compound is the first which has been observed to induce active regression of the Walker carcinoma even when the tumour has become well established. However, its effects upon the mouse tumours mentioned is by no means proportional, some indeed being relatively resistant. All these

(a)

(b)

FIG. 1 Comparison between (a) the normal appearance of the Walker rat carcinoma and (b) the changes induced by treatment with N,N-di(2-chloroethyl)-p-aminophenylbutyric acid.

[To face page 193]

of the mechanics of mitosis, and, not infrequently, great increase in cell size (Fig. 1). In the effects which it produces on the course of body growth in the rat, and on the red cell, lymphocyte and neutrophil counts of the peripheral blood, *NN*-di(2-chloroethyl)-*p*-aminophenylbutyric acid presents an interesting contrast with Myleran, and the combined effects of the two agents when given together, or super-imposed, are highly similar to the characteristic changes produced in the same elements by X-radiation, as is shown in Fig 2. This aspect has been subjected to a specially detailed study by Dr. L. A. Elson, with results which promise to be of service in elucidating, in chemical terms, the mechanism of action of X-radiation itself.

Because of its rather exceptional inhibitory activity when tested against various tumours experimentally, *NN*-di(2-chloroethyl)-*p*-aminophenylbutyric acid was selected for clinical trial in cases of malignant disease for which no better or no other therapy was indicated, and with special reference to the lymphomas and lymphatic leukæmia This trial was conducted by Dr. D. A. G. Galton and Dr. M. M. Till, to whom I am indebted for the facts on which the following account is based Although the trial has been in progress for no longer than a year, a tentative or preliminary statement can at least be made To date, 28 cases have been treated, and may be broadly grouped as follows:—reticulum-cell sarcoma four, lymphosarcoma six, Hodgkin's disease nine, follicular lymphosarcoma three, chronic lymphatic leukæmia two, mycosis fungoides one, multiple myeloma one, acute lymphatic leukæmia one, acute myeloid leukæmia one. Especially in the first three groups there is some overlap both clinically and pathologically, and hence it is unprofitable to attempt to assign any therapeutic effects too rigidly to one or another. One case of reticulum-cell sarcoma, three of lymphosarcoma, and one of Hodgkin's disease and of multiple myeloma, were in the terminal stages of disease and received treatment for only one to four weeks. Two showed some regression, to a striking degree in one case of lymphosarcoma, but of no real

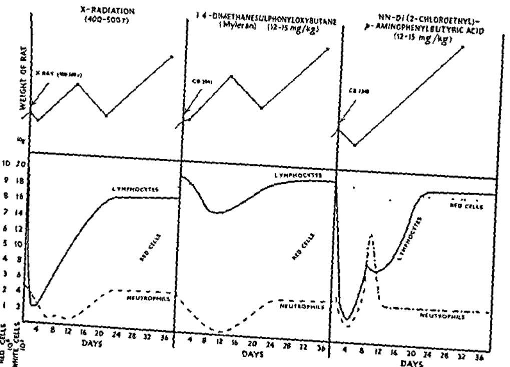


Fig. 2 A comparison between the effects characteristic of (a) X-radiation; (b) 1 4-dimethanesulphonyloxybutane ('Myleran'), and (c) *NN*-di(2-chloroethyl)-*p*-aminophenylbutyric acid, upon body growth and the red cell, lymphocyte and neutrophil counts in the peripheral blood of the rat. (L. A. Elson.)

therapeutic significance. Seven cases with progressive generalized disease and serious constitutional disturbance were treated, all considered to be beyond the compass of chemotherapy. Of these, six showed some response, in that persistent pyrexia subsided, sweats ceased, and lymph node masses regressed. In all cases but one, however, these effects were shortlived, and cannot be considered therapeutically useful. The exception is a woman with generalized reticulum-cell sarcoma, who presented with a short history of sore throat, sweats, malaise, loss of weight and intense pruritus, and who on examination was febrile, with enlarged ulcerated tonsils, generalized massive lymphadenopathy, hepatosplenomegaly, and skin rash. Four days after starting a course of CB 1348 by mouth she was afebrile, and thereafter her symptoms rapidly subsided and all foci of disease regressed dramatically She is well and recurrence-free, seven months later. Instances of the kind occur from time to time with all remedies of this general class, but they are exceptional and not in themselves important in assessing the value of any given agent, which must rather be judged by its average performance.

Ten cases were treated for generalized disease of more recent onset or of apparently greater chronicity than those already considered. They include one case of lymphosarcoma, three of follicular lymphosarcoma (a more differentiated and sometimes more slowly progressive form), and six of Hodgkin's disease. All except one responded in some degree, although only in the cases of follicular lymphosarcoma was the effect at all striking. It must be said at once that follicular lymphosarcoma is the most treatment-sensitive of this group of diseases, and good results can be obtained with diverse methods. Nevertheless, the smooth response, and the absence of side-effects or of serious damage to the bone-marrow, have been gratifying features in these three cases, particularly since one had had repeated treatment over the past seven years with X-radiation, triethylenemelamine and ^{32}P, with poorly functioning bone-marrow in the last year. A case of mycosis fungoides, of four years' duration, was treated after having

become refractory to radiotherapy and having failed to respond to ^{32}P. Remarkable improvement followed, but within a month relapse again occurred in spite of increased dosage.

In chronic lymphatic leukæmia, therapeutic assessment is specially difficult because of conflicting opinion concerning treatment policy and general management. Although both cases treated were in the later stages of the disease, neither had received previous radiotherapy. Both responded well, and mention can be made of the smoothness of the remissions and freedom from side-effects.

In all cases save two the drug was administered orally, first in a dose of 0·2 mg. per kg. body weight per day, and later, on account of the occasional observation of moderate neutropenia after four to six weeks, at half this level. The average total dose was 400–600 mg. over a period of four to eight weeks, although a few cases received the drug for longer periods—in one case for sixteen weeks, representing a total dose of over 1 g It may be noted that the bone-marrow of patients in the lymphoma group is more sensitive than that of other persons both to X-rays and to the nitrogen mustards. Within the lymphoma group, lymphatic leukæmia cases show the most sensitive marrow, followed closely by follicular lymphosarcoma cases; patients with Hodgkin's disease and the lymphosarcomata tolerate rather larger doses. Although in the present trial any depression of the bone marrow has been mild and quickly reversible, (possibly on account of the use of sub-optimal doses), it must on no account be supposed that the compound is entirely safe. Thus it is capable of producing destruction of all the bone marrow elements, with consequent aplasia, and repeated small doses, in the rat, may lead eventually to myelosclerosis with splenomegaly. Control of treatment by frequent blood counts must therefore be considered essential.

From the experience so far gained there would appear to be little doubt of the efficacy of *NN*-di(2-chloroethyl)-*p*-aminophenylbutyric acid in cases of follicular lymphosarcoma and of chronic lymphatic leukæmia. How the long-term results

will compare with those obtained from radiotherapy still remains in doubt, and can only be determined from the study of larger numbers. Although the general trend has not greatly differed from that experienced with the aliphatic nitrogen mustards or triethylenemelamine, the results themselves have certainly been of sufficient promise to warrant further trial: even if the drug is unlikely to prove inherently superior to these and others already used, it will still retain the real advantage of greater safety.

The original purpose of this Conference was to consider present trends and future policies in leukæmia research. Gratifying though it may be that the work of the past ten years has yielded therapeutic agents, even if these are of limited power and applicability, there seems little doubt that the future cannot lie with cytostatic and mitotic poisons, or with radiomimetic agents. Sir Lionel Whitby, Dr. Furth and Professor Engelbreth-Holm have all testified to our real need, namely, a knowledge of the processes of differentiation, and of the normal regulation of growth, sufficiently profound to allow, in the case of the cancer cell, a restoration of function—a healing of the biochemical lesion, rather than the mere destruction of the cell which carries it. I propose, therefore, to proceed to the thesis that the more fundamental study of agents of the kind with which I have been dealing will lead to entirely new conceptions which may, as it were, by-pass the present situation and so provide a more physiological solution. Most of the types of chemical agent to which I have referred are carcinogenic and mutagenic Their high degree of chemical reactivity, the dependence of activity on a polyfunctional structure, and the ease with which the relation between structure and activity can be studied, have led to the conception of their action through biological alkylation, whether of genetic protein or its precursors, in the case not only of the mustards but of the epoxides, myleran series, and polyethyleneimines as well From their effects on the salivary gland chromosomes in *Drosophila*, Dr O G Fahmy has concluded that these chemical agents produce a disabling

effect on some of the gene loci, the affected gene failing to reproduce itself either completely or only partially. Here may be a demonstration that such agents are capable of producing genetic loss, and one consistent with the view from other evidence, that in the cancer cell, or the leukæmia cell, the loss may be of some controlling enzyme system normally regulating the synthesis or availability of substances essential to division. Our own thinking along these lines has been greatly influenced by much independent work on the possible *rôle* of xanthopterin in the regulation of kidney growth and size. In this case the active pterin is a substrate for xanthine oxidase, and clearly it is not beyond the bounds of possibility that somatic mutation, through the loss or modification of some such regulatory enzyme protein, could readily lead to the accumulation, or unrestricted synthesis, of substances vital to cell division and determining its rate. Nor would this interpretation be inconsistent with evidence already existing, mainly from the work of the Madison school, as to the deficiency of enzymic systems in the mitochondria of the cells of tumours produced by dimethylamino-azobenzene, or the elimination of protein as an essential stage in hepatic carcinogenesis. It may indeed be possible to proceed further, and to hazard a guess that the ultimate phase in the treatment of leukæmia may be some sort of substitution therapy, involving not the cytostatic and radiomimetic agents we have so far been considering, nor indeed artificially constructed purine, pteridine and folic acid antagonists, but naturally occurring enzymes normally concerned in the regulation of purine, pteridine, folic acid and nucleic acid synthesis and metabolism, in which the leukæmic cell may conceivably be deficient through a process of carcinogenesis by loss.

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[Discussion of this paper was postponed until after the paper by Dr. Hansen.—Ed.]

CLINICAL EXPERIENCE WITH MYLERAN THERAPY, ESPECIALLY IN MYELOID LEUKÆMIA

POUL BJERRE HANSEN

This report is a brief survey of the results obtained in an eighteen-month clinical trial of "Myleran" in chronic myeloid leukæmia and some allied disorders. The Radium Hospital in Aarhus received the drug from the Chester Beatty Research Institute in March 1952, and treatment was given on about the same lines as in the clinical trial which was commenced at the Royal Cancer Hospital in 1951 by Dr. Galton.

During this period of eighteen to twenty months about 60 patients were treated with Myleran. Table I shows the distribution of the patients according to diagnosis.

Table I

CASES TREATED WITH MYLERAN, MARCH 1952—OCTOBER 1953

Chronic myeloid leukæmia	33
Subacute myeloid leukæmia	11
Chronic lymphatic leukæmia	3
Chronic aleukæmic lymphatic leukæmia combined with "Macroglobulinæmia"	4
Multiple myeloma	3
Polycythæmia	2
Hodgkin's disease and lymphosarcoma	3
	59

As a beneficial effect was obtained predominantly in patients with chronic myeloid leukæmia, this first main group will be considered in some detail. The ages of the patients ranged from eleven to seventy-four years, there were 16 women and seventeen men. Fifteen of the 33 patients had previously received radiotherapy (up to seven courses), chemotherapy

(arsenic, urethane, colchicine, etc.) and other forms of therapy. The average duration of the disease before Myleran treatment was about seven months in the 18 previously untreated patients, and about twenty months in the 15 previously treated patients. The average ages of the patients in these two subgroups were roughly identical, namely about 45 and 47 respectively, when Myleran therapy was started.

A summary of the results obtained by Myleran therapy in these 33 cases is given in Table II.

Table II

RESULTS OF MYLERAN THERAPY IN 33 CASES OF CHRONIC MYELOID LEUKÆMIA

Period of observation (months)	*Initial and maintained remission*		*Initial remission followed by resistance to Myleran and relapse*				*No response (rapid progress or terminal relapse)*	
	All alive		*Alive*		*Dead*		*All dead*	
	*	†	*	†	*	†	*	†
0–2	1	0	0	0	1	0	3	2
3–5	2	0	0	0	0	3	0	0
6–8	2	0	1	0	0	0	1	0
9–11	2	2	0	1	0	0	0	0
12–14	2	1	0	0	1	1	0	0
15–17	0	0	0	1	0	1	0	0
18–20	2	3	0	0	0	0	0	0
	11	6	1	2	2	5	4	2
	12 * & 6 †			20	6 * & 7 †			13

(18 previously untreated cases *)
(15 previously treated cases †)

Of the 18 patients who had not received any previous treatment, 12 are alive; in one of them "resistance" to Myleran has developed, and the disease is again in progress after an initial remission lasting five to six months. So far,

11 patients show excellent remissions having lasted from five to twenty months in ten cases. The remaining six patients in this group died. In two instances an initial response to Myleran treatment was seen. In the first of these, a promising remission was interrupted about two months later by a fatal cerebral hæmorrhage, and in the second a good remission lasting ten months was superseded by an acute terminal relapse. No response to Myleran treatment was seen in the last four patients; the disease advanced rapidly and the patients succumbed, three of them within one to two months. It was felt that in one or two of these cases radiotherapy might, perhaps, have been more beneficial, but as far as the remaining cases are concerned, we are of the opinion that Myleran therapy was superior to, or at least as efficient as radiotherapy would have been. Obviously, this is only an impression based on personal experience with both kinds of treatment. A contemporary control trial with radiotherapy has not been attempted, so far. During the period of Myleran trial only two patients with myeloid leukæmia received radiotherapy.

The results obtained in the 15 previously treated patients, seem, however, to lend some support to this opinion Of these 15 patients, six are alive and show good remissions which have lasted from ten to twenty months. Two other patients responded well to Myleran therapy and had remissions lasting ten and fifteen months, but then an untreatable, presumably terminal relapse developed, and the patients are in a very poor condition (Nov 1953). Of these eight patients so far mentioned, seven had previously received radiotherapy, and at least five were definitely resistant. The remaining seven patients died. In three cases a good immediate response to Myleran was seen, but only transient remissions lasting a few months were obtained All three patients were resistant to radiotherapy. Two other patients showed good and relatively long remissions lasting thirteen and sixteen months, followed by an acute relapse. In the last two cases no response to Myleran treatment was obtained One of them died from an

acute, profuse retroperitoneal hæmorrhage, before any definite response to Myleran could be expected. These four patients had previously received two to three courses of radiotherapy with moderate benefit only.

This brief survey, giving merely a rough outline of the fate of the patients treated with Myleran, is based on thorough

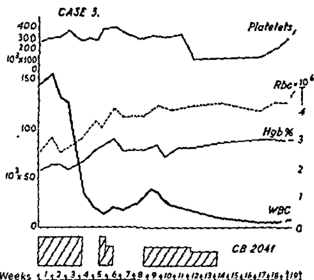


FIG 1. Case 5 Sixty-two-year-old woman Radiotherapy and colchicine treatment previously. Good initial response to Myleran therapy (March, 1952) A satisfactory remission is still maintained with a Myleran dose of 10–12 mg per week

examinations of the patients before, during, and after the treatment; all details are omitted. Only two illustrative curves of the typical hæmatological changes in two patients who responded well to Myleran therapy will be shown (Figs. 1 and 2). In addition, a typical example of the regression of an enlarged spleen is shown in three photographs, one taken just before Myleran treatment was started, and the others three and five weeks later. Finally, it must be emphasized that just like all other therapeutic measures so far available in chronic myeloid



Fig 3



Fig. 4

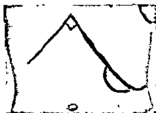


Fig 5.

acute, profuse retroperitoneal hæmorrhage, before any definite response to Myleran could be expected. These four patients had previously received two to three courses of radiotherapy with moderate benefit only.

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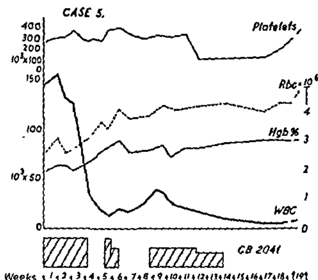


FIG. 1. Case 5. Sixty-two-year-old woman. Radiotherapy and colchicine treatment previously. *Good initial response to Myleran therapy (March, 1952). A satisfactory remission is* still maintained with a Myleran dose of 10–12 mg per week.

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Fig 3

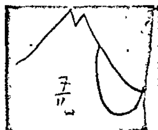


Fig 4

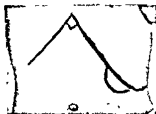


Fig 5

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leukæmia, Myleran treatment must be regarded as palliative and must therefore follow the lines of all palliative therapy, which means that, first of all, it is the general condition of the patient that counts.

The second main group treated with Myleran consisted of 11 patients with subacute myeloid leukæmia. The ages of

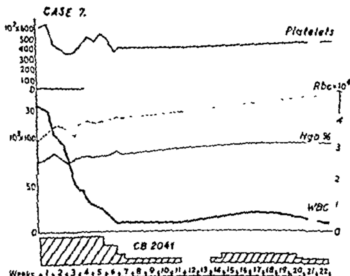


FIG 2 Case 7 Forty-one-year-old woman. No previous treatment Excellent initial and continued response to Myleran therapy The remission obtained in April, 1952 is still maintained with a Myleran dose of only 3–4 mg per week

... ranged from seventeen to ...

had not received any previous treatment. Myleran therapy was tried, but no real response was obtained in any of these cases In two or three cases a very slight and brief improvement was seen, followed by rapid progress of the disease, and all the patients died.

It seems quite obvious that Myleran therapy is ineffective in cases of subacute myeloid leukæmia and in the terminal phase of the disease. In view of the discouraging results obtained in subacute cases, no attempts were made to try Myleran therapy in acute leukæmia.

In the various smaller groups of allied diseases, Myleran proved quite ineffective in chronic lymphatic leukæmia, Hodgkin's disease, and lymphosarcoma. There were three cases of multiple myeloma, and it was felt that two of these patients derived remarkable benefit from Myleran, particularly as a maintenance therapy. In one case of uncomplicated polycythæmia no effect of Myleran was recorded; in another case complicated by a myeloid leukæmic reaction, a distinct "remission" was seen. In the last group consisting of four patients suffering from the peculiar aleukæmic "lymphatic leukæmia" combined with macroglobulinæmia a certain improvement in the general condition was obtained, and it was possible to maintain this "remission" for a considerable time in two cases. No influence on the neutropenia or on the very high sedimentation rate and the abnormal serum globulin values (measured by paper-electrophoresis and ultracentrifugation) has been recorded. These patients are still under maintenance treatment.

Finally, the dosage problems in Myleran therapy deserve brief mention. Myleran was given orally in 2 mg. tablets. The "normal" or average schedule was 6 mg. daily for four to six weeks, followed by smaller maintenance doses, for example, 2 mg. daily at first and then 2 mg. every second day, or sometimes only one or two tablets per week. Sometimes, treatment was stopped for one or two months, and in other cases the dose had to be increased periodically, for example, to five tablets (10 mg.) per week This procedure was continued for months. The main guide in decisions as to treatment and dosage has always primarily been the general condition of the patient, including the hæmoglobin level, and secondarily the white blood counts, the size of the spleen and lymph nodes etc. Blood counts are indispensable as a control

measure to avoid a too pronounced depressive effect on the hæmatopoiesis, especially on the thrombopoiesis. The administration of Myleran is easy and, apart from thrombocytopenia, there are no side-effects. Usually, the treatment is started in hospital and continued there for the first four to six weeks; then the maintenance therapy is carried on with the patients staying at home, and in many cases, working normally. The patients return for follow-up examinations at least once a month, while blood counts are done weekly (or nearly so) in collaboration with local hospitals.

In a relatively high proportion of the cases treated, several blood transfusions were given in the initial phase of treatment, because many of the patients were in a very poor condition when first seen; quite often the hæmoglobin level was below 40 per cent Later, no auxiliary therapy was necessary in the majority of cases. In a few patients, combined therapy with urethane and Myleran in small maintenance doses was attempted. The reason for this combined therapy was that, with the Myleran dosage used, it was not possible in any of the cases to deplete the peripheral blood completely of immature cell forms. This was not constantly achieved either with this supplementary therapy, but in two cases where the tendency to thrombocytopenia was disquieting, a permanent combination of urethane and Myleran therapy seemed to be rather useful. These two patients are both alive with good remissions. The possibilities of combined therapy of all kinds ought to be subjected to further investigation in future.

The work performed in the hæmatological and research laboratories at the Radium Hospital in Aarhus, Denmark, is aided by grants (to J Bichel) from The Tata Memorial Foundation, The Anders Hasselbalch Anti-Leukæmia Foundation and C P. Schepler and Wife's Bequest, the Irma Foundation, and is supported by the Danish Anti-Cancer League Thanks are also due to the Chester Beatty Research Institute for the supply of Myleran and kind collaboration

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away precipitously.

Table B shows the growth-inhibitory effect of different doses of

Table B (HADDOW)

ACTION OF MEMBERS OF THE SERIES ON GROWTH OF WALKER RAT CARCINOMA 256

(Each experiment based upon ten rats)

Dose (mg per rat of average weight 200 g)	n								
	2	3	4	5	6	7	8	9	10
2	—	—	+	+	+	—	—	—	—
5	—	—		·	+	+	+	—	—
10	—	+						+	—

each compound in the same series. Again, the compounds with four, five or six carbon atoms in the central chain are the most effective by dose. In separate work by Dr. Ross and Mr. Timmis on the chemical

tumour, and toxicity.

WARWICK Prof Haddow mentioned that greater effects had been noted on the Walker carcinoma, with *NN*-di(2-chloroethyl)-*p*-aminophenylbutyric acid (CB 1348) than had been seen before with other members of the mustard series. Have you tried this compound in

(CB 1348) in the lymphoma group, Dr M Till and I carried out an extensive trial on cases of advanced carcinoma of varied origin, mostly with widespread metastases. Although we obtained interesting information about the effects of the substance on peripheral blood and bone marrow, and on its general toxicity, there was no

DISCUSSION

ISRAËLS: Would Prof. Haddow like to tell us what directed his attention to Myleran? It is quite different chemically from the other

Thus we accumulated a number of pairs of compounds, one having

Loveless and Ross then suggested that the polyfunctional character might be required in order to effect chemical cross-linkage between contiguous linear macromolecules, possibly in the chromosomes themselves. This interpretation we now know to be unduly simplified, but

and which includes 'Myleran' (1·4-dimethanesulphonyloxybutane), was happily an exception, and represents, so far as I know, the only example in this particular connection, in which compounds elaborated on what

generally in normal animals the mustards hit the lymphoids much more. I wonder if Prof. Haddow has any more experimental data on that, because I think that its effect in normal animals on the two different types of cells is very important.

HADDOW: I had intended to mention this if there was enough time Table A is based on work done by Dr. Elson, and shows the effect on the

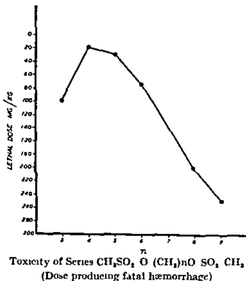
Table A (HADDOW)
EFFECT OF SERIES $CH_3SO_2O-(CH_2)_n-OSO_2CH_3$ ON CIRCULATING NEUTROPHILS IN THE RAT

n	Dose mg/kg (D)	Per cent fall in neutrophils from normal (F)	$\frac{F}{D}$
3	40	50	1·3
4	8	70	8·8
5	12	85	7·1
6	40	60	1·5
7	60	40	0·7
8	80	50	0·6
9	100	40	0·4

count alone.

DUSTIN: From my very limited experience Myleran is an excellent treatment for myeloid leukæmia, and the patients feel much better.

Dr. Hansen mentioned the association of urethane and Myleran, and



Toxicity of Series $CH_3SO_2\,O\,(CH_2)_nO\,SO_2\,CH_3$
(Dose producing fatal hæmorrhage)

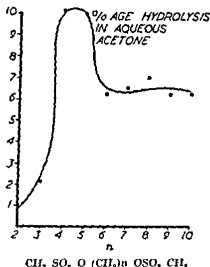
FIG 2. (Haddow)

I would like to know what are the doses of these two drugs, when they are used together.

HANSEN: In one of the two cases I mentioned the Myleran dose was

twice as much intravenously.

WHITBY. I'd like to look at this problem from another angle just for a moment. What exactly do we mean by a good clinical response or a good remission? Should it be synonymous with a reduction in the leucocyte count? I noticed that a number of Dr. Hansen's cases had ample hæmoglobin and other factors, all they were suffering from was

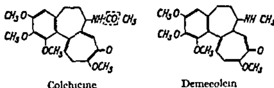


$CH_3\ SO_2\ O\ (CH_2)n\ OSO_2\ CH_3$

FIG. 1 (Haddow).

blood.

this purpose a group of 17 rabbits and six cats were used. After preliminary experiments, the cats were given 0·25 mg. per kg. body weight daily intraperitoneally and the rabbits 3 mg. per kg. intravenously. This dosage was chosen to produce a definite change in the blood picture without



Colchicine Demecolcin

causing any significant side effects. Erythrocytes, reticulocytes and leucocytes were counted in all the animals, differential counts were prepared, and periodic marrow punctures performed.

Only the main findings will be indicated here (Fig. 1). For more details the reader is referred to the dissertation by Lichtman (1954).

Two days after the intravenous injection of Demecolcin the total number of leucocytes began to drop, due to a decrease in the neutrophils. The effect was delayed about twelve to fourteen days in animals treated orally After cessation of therapy, the neutrophil count began to rise in two days, attaining its initial value within eight to ten days. With the dosage used, the lymphocytes were found to be only slightly affected in the cat and negligibly in the rabbit, with similar results in the thrombocyte, erythrocyte and reticulocyte counts. Examination of the bone marrow revealed a definite diminution to approximately one-third of the initial values of the myelogenous elements, so that the erythropoietic portion appeared relatively increased. During the administration of this rather high dose no side effects were observed in the rabbit other than a definite weight loss and an inhibitory effect on spermatogenesis. The cats were more sensitive, and were unable to tolerate a dosage of 0 5 mg. per kg. body weight,

A NEW COLCHICINE DERIVATIVE (DEMECOLCIN CIBA) AS A CYTOSTATIC AGENT IN MYELOGENOUS LEUKÆMIA

S. MOESCHLIN, H. MEYER and A. LICHTMAN

SINCE the work of Jacobj (1890), Dustin (1938) and Bucher (1939), the inhibitory action of colchicine on mitosis, with blocking in the metaphase, is well known. With the exception of local application to skin tumours, its clinical use by Landolt (1943–44) in the therapy of tumours and leukæmias failed, due to the generalized toxicity of the substance. Santavy and Reichstein (1950) succeeded in isolating purified alkaloids from the mixture present in *Colchicum autumnale.* Among these an alkaloid designated as Demecolcin (deacetylmethylcolchicine) proved of interest because of its lowered general toxicity. The CIBA Company kindly placed this drug at our disposal in the autumn of 1952 for use in animal experimentation and in clinical trials as a therapy for leukæmias and other neoplasms ("Colcemid", CIBA).

Pharmacological Properties

Demecolcin is a crystalline, water-soluble alkaloid obtained from the corm of the *Colchicum autumnale* plant. The normal acetyl group is replaced by a methyl group. M. P. 180–186°C. Schar, Loustalot and Gross (1954) observed an antimitotic action similar to that of colchicine in cultures of normal and malignant cells and in implanted tumours. However, the acute lethal intravenous dosage for mice was 30–40 times higher than for colchicine

Animal Experimentation

Prior to clinical use it was important to study the action of Demecolcin on the peripheral blood and bone marrow. For

. . . . . . ' ' æmia. Since the initial to be carefully studied, lts of a few cases which have been observed for a long time. The results obtained have been so impressive that we felt it would be of practical interest to submit a preliminary report.

(a) Chronic myelogenous leukæmia (ten cases)

A distinct depressive action was seen on the pathologically increased leucocyte levels concomitantly with a decrease in the size of the enlarged spleen. At the same time, there was an increase in the number of erythrocytes and in the hæmoglobin values A comparison of the sternal marrow and splenic puncture prior to therapy and after three months of treatment, showed a marked change from the immature myeloid forms to more mature forms. This change is similar to that described in our investigations of arsenic compounds and urethane (Moeschlin, 1951).

The clinical course of the forty-seven-year-old male (W.) shown in Fig 2 may be taken as typical of this disease under Demecolcin therapy in our series. He has now received ambulatory treatment for thirteen months with periodic controls Under a maintenance dose of 2–5 mg. of Demecolcin daily he has been enabled to return to full-time employment.

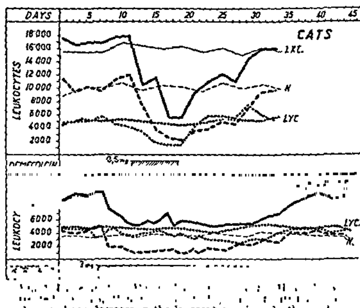
(b) Acute and subacute myeloid leukæmia (six cases)

Although our knowledge here is only in the preliminary stages, the results obtained have been so encouraging that further clinical trial of Demecolcin in acute myeloid leukæmia is strongly indicated.

The clinical course of a thirty-nine-year-old male (Sch.) with chronic myeloid leukæmia showing a subacute myeloblastic episode, is seen in Fig. 3 Two other cases of acute terminal blastic episodes in chronic myeloid leukæmia also showed a very good respopse The leucocytes dropped from 114,000 to 17,000, with the disappearance of blast forms, and

showing emesis, ptyalism, muscular pain, and difficulty in swallowing. No more severe changes were seen in the bone marrow of cats than in the rabbits; this is in contrast to our findings in cats given urethane (Moeschlin and Bodmer, 1951).

In summary, the doses used, namely 0·25 mg. per kg. in cats, and 3 mg. per kg. in rabbits, produced a definite, almost



of the drug

elective inhibition of the granulocytopoiesis without any important side effects.

Clinical Tests

Because the animal experiments demonstrated an impressive selective inhibition of the granulocytopoiesis, with a limited toxicity, we were interested in trying this drug in

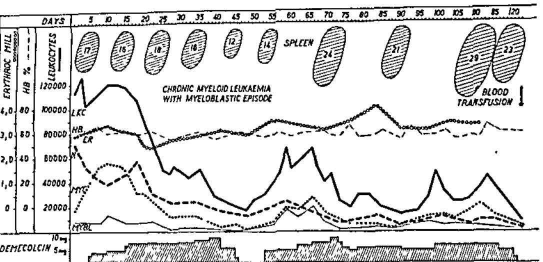


FIG. 3. Demecolcin treatment of a *chronic myelogenous leukæmia with a terminal myeloblastic episode*, who was admitted in a very serious condition. Clear effect on the disease, with a drop in the myeloblast and myelocyte values and decrease in the size of the spleen. Short interruption of therapy caused a quick relapse and increased myeloblast values. Patient was discharged in good condition with a daily maintenance dose of 3-5 mg. Two other cases of chronic myelogenous leukæmia with a terminal myeloblastic episode showed a similar result.

Fig. 2 *Therapy of chronic myelogenous leukæmia with Demecolcin.* The marked therapeutic success is easily seen from the drop in leucocytes and regression in the size of the spleen. There is a simultaneous rise in the erythrocyte count and hæmoglobin. The intercurrent rise in leucocytes was due to a tooth abscess. With a maintenance dosage of 2–3 mg daily, the patient returned to work and has experienced no side effects *during* treatment for thirteen months.

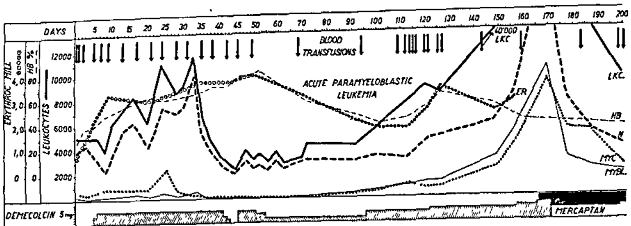


FIG. 4 *Acute paramyeloblastic leukæmia*, subleukæmic form Bone marrow showed principally paramyeloblasts and promyelocytes. On admission, patient was in poor general condition Under Demecolcin therapy and blood transfusions, there was a definite slowing of the course of the leukæmia, but no complete remission Marrow remained 50 per cent myeloblasts and promyelocytes. With 5 mg Demecolcin daily he remained in good physical condition for five months and returned to work. Marrow still contained 50 per cent myeloblasts and promyelocytes, however A relapse in the fifth month responded to mercaptopurine. The patient died twelve months after the onset of the disease

the enlarged spleen and lymph glands showed a decided decrease in size under a daily dose of 10 mg. Punctures of these organs had previously shown up to 90 per cent blast forms in the smears. The patient's appetite and general condition improved so markedly that he was discharged from the hospital. Unfortunately he died four months later of a traumatic splenic rupture incurred in a severe accident. Autopsy showed azoöspermia in the testes. The two other cases are still in good condition.

The case of a thirty-three-year-old male (B.) with acute paramyeloblastic leukæmia is presented in Fig. 4. The patient first developed symptoms in March 1953, exhibiting a hæmoglobin of 44 per cent and 1·2 million erythrocytes. He was hospitalized on April 7th of the same year.

[illegible] 17,100. Sternal marrow showed 50 per cent blast forms with large nuclei and relatively little cytoplasm.

[illegible]

A resistance to Demecolcin developed in the fifth month, manifested by a new rise in the leucocytes and blast forms, with the simultaneous appearance of a thrombocytopenic purpura. Mercaptopurine (= Purinetol) was instituted (Fig. 4), resulting in a new remission. The patient died twelve months after the onset of his disease.

(c) Chronic lymphatic leukæmia

On the basis of the animal experiments, the main action of Demecolcin was expected to be on the granulocytopoiesis and not on the lymphopoiesis. This was confirmed in our clinical trials in the treatment of lymphatic leukæmia. *The drug should therefore not be used in such cases.*

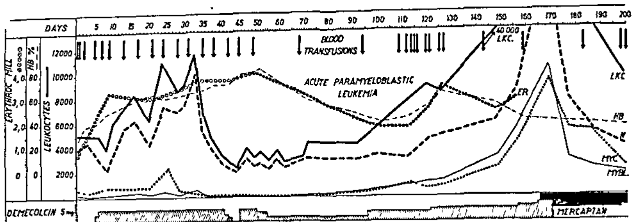


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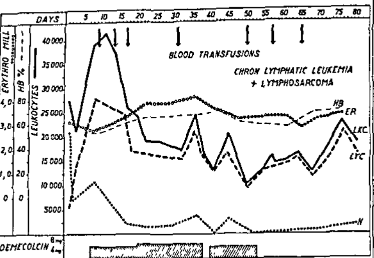
The clinical results are shown in Fig. 5. The first patient, a seventy-two-year-old male, had a diagnosis of typical lymphatic leukæmia (old lymphocytic cell type) combined with lymphosarcoma, exhibiting extremely pathological cells in the gland punctures. There was a good response in the treatment of the lymphosarcoma, attended by a slight decrease in the lymphocytes, but he developed an agranulocytosis of ten days' duration, necessitating blood transfusions and antibiotics.

The second patient was a fifty-seven-year-old male, who was admitted to the hospital in an extremely poor general condition. Blood values were: 1·7 million erythrocytes with 89 per cent hæmoglobin; leucocytes 190,000, with 96 per cent lymphocytes. Sternal puncture revealed infiltrated marrow. After receiving only 65 mg. over a period of thirteen days, he died in an acute lymphatic exacerbation with a terminal cell rise to 400,000. He also developed a severe necrotic tonsillitis.

(d) Carcinomas and sarcomas

A total of 12 cases were treated. The daily dosage varied from 3 to 7 mg. Side effects were granulocytopenia (three of the 12 cases), with a drop of the granulocytes in one case to 390 (total: 870 leucocytes) (Fig. 6). No other toxic effects were observed. Although nausea and emesis were present in three cases prior to therapy none could be traced solely to the Demecolcin. As a control, a patient who tolerated Demecolcin well was given three doses of 1 mg. of ordinary colchicine, whereupon severe diarrhoea and nausea promptly appeared on the third day.

A definite therapeutic effect was not observed in any of these cases, although a more normal temperature and an obvious increase of appetite occurred in a case with widespread hypernephromatous metastases to the lungs and bones. There were also slight recessions in lymph node swelling in two cases of lymphosarcoma, which may be interpreted as cytostatic inhibition. However, since these changes have been



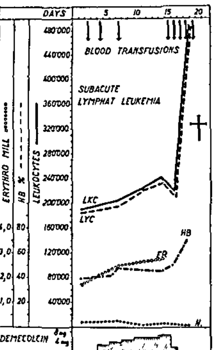


FIG 5 Failure of Demecolcin therapy in *lymphatic leukæmia* LEFT GRAPH *chronic lymphatic leukæmia with concomitant lymphosarcoma.* Subjective feeling of improvement. Increased appetite and moderate decrease in lymphocytes after a rise due to the initial stimulating effect of the drug. RIGHT GRAPH. *subacute lymphatic leukæmia and very poor general condition; a fatal exacerbation occurred under the Demecolcin therapy* There was a rise in the lymphocytes with concurrent drop in thrombocytes Demecolcin is therefore contra-indicated in the treatment of lymphatic leukæmias.

noted spontaneously in this type of neoplasm, the actual therapeutic effect is difficult to evaluate.

Unfortunately, due to a lack of cases, the preparation has not yet been tried in any cases of acute gout.

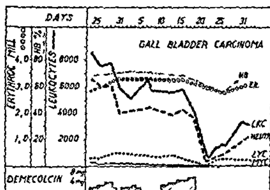


Fig. 6. *Granulocytopenia occurring during Demecolcin therapy of a gall bladder carcinoma* Carcinoma remained uninfluenced, as did other carcinomas and sarcomas in our series.

Discussion of the Findings

The isolation of Demecolcin (Santavy and Reichstein, 1950) from the mixture of alkaloids present in *Colchicum autumnale* provided for the first time a colchicine derivative retaining its cytostatic action with 30 times less toxicity, so that it could be used for experimental and clinical trials.

Our animal experiments showed a clear, almost elective inhibitory action on granulocytopoiesis (see Fig. 1). Depression of erythro- and lymphopoiesis only occurred with higher doses. Although the investigations of patients with carcinoma showed no definite effect on the clinical course of the carcinoma, or in the sarcoma cases, a slight to definite decrease in the granulocytes was noticed. In one case, a definite agranulocytosis developed, with a drop in the granulocytes from 7095 to a level of 390. No other effects besides slight alopecia in two cases were noted with the daily dosage of 3–7 mg. used in this series.

On the basis of its almost elective action on the granulocytic elements, a blocking action which is quickly reversible, the next step obviously was to try it on myelogenous leukæmias, as had been done previously by Landolt (1943–44) with colchicine. Although the number of treated cases is still small, our observations have shown that there is no doubt about its therapeutic effect. In all ten cases of chronic myelogenous leukæmia cited, it was possible to bring the blood picture almost to normal, at the same time achieving an improvement in the general well-being of the patients. A definite decrease in the size of the spleen was also achieved. It should be pointed out that the case which showed a transition into the acute form (7400 myeloblasts in the peripheral blood and prominence of myeloblasts in the node and splenic punctures) must also be considered a subacute myelogenous leukæmia.

Meanwhile similar results in more than 30 cases of chronic myelogenous leukæmia were obtained independently by Bock in Tubingen (personal communication).

The clear therapeutic success in chronic myeloid leukæmia made us consider using the drug also in a primary acute subleukæmic leukæmia. This case, No 3 (Fig. 4), who was treated with 5 mg. of Demecolcin daily, was restored to a good general condition and enabled to return to work He was maintained in this condition for a period of five months The blood picture was partially normalized but the marrow still maintained 48–57 per cent pathological myeloblasts and promyelocytes. After five months, resistance to Demecolcin developed, and Mercaptopurine gave a new remission lasting four months; the patient died twelve months after the onset of the disease.

Cautious as one must be in the evaluation of therapeutic successes with acute leukæmias, we believe the improvement in case No. 3 can be regarded as a result of treatment with the colchicine derivative. *Further work is required to see if* it will prove of value with other acute leukæmias Whether any effect can be expected in the completely undifferentiated leucoblastic forms is still a matter of conjecture. Its use in a

case of lymphosarcoma produced only equivocal results; and in a case of Hodgkin's disease as well as in two cases of multiple myeloma, it produced no change at all. An attempt to use Demecolcin in lymphatic leukæmias showed that its use here is strictly contra-indicated. In case No. 4, there was an initial improvement, with increased well-being and a drop in the number of lymphocytes (Fig. 5). However, subsequently the granulocyte count dropped even faster, so that for a time an absolute granulocyte count of 74/mm.^{3} existed. Another patient admitted in an advanced state of subacute lymphatic leukæmia suffered an acute exacerbation and died under the Demecolcin therapy. The terminal lymphocyte count rose from 200,000 to 400,000 with the simultaneous appearance of numerous lymphoblasts. It is difficult to say to what extent the drug was responsible. Similar exacerbations have occurred after X-ray irradiation of these diseases. Since all these cytostatics produce an initial stimulating effect prior to inhibiting the mitotic process, it is possible that that is what occurred here. On the basis of these observations, *we wish to emphasize once again that the use of Demecolcin is strongly contra-indicated in the treatment of lymphatic leukæmia.*

Dosage

Dosage in chronic myelogenous leukæmia: initial oral dose of 3×1 mg. daily, increased after three days to a total of 7 or 8, and possibly 10 mg. per day. With repeated leucocyte counts, this dosage is maintained until the leucocytes drop to 30,000, at which time the medication is stopped for a period of three days. This is done because the leucocytes continue to drop to low levels A maintenance dose of 1–4 mg. daily is established and periodic blood counts done, at first at intervals of three days, but the time can be lengthened to once a month. The individual maintenance dose varies from case to case. With cessation of therapy the leucocytes rise quickly within six to eight days, which is quicker than with arsenic or urethane. Resumption of the therapy shows an effect after a latent period of about fourteen days. That is

why continual therapy with small doses appears better than intermittent use of the drug. In severe cases the intravenous daily administration of 5 mg. is advised, which gives an earlier response.

Dosage in acute myelogenous leukæmia: initially 3 mg. Demecolcin daily, with slow increase to 5 mg., combined with blood transfusions and an antibiotic prophylaxis of 600,000 i.u. of penicillin and 0·5 g. streptomycin intramuscularly daily.

Side effects. no subjective side effects were observed with the dosages given here. Absence of the gastro-intestinal symptoms usually seen with colchicine is particularly emphasized As with other cytostatics (nitrogen mustard, TEM, Myleran, arsenic etc.), there is an inhibition of spermatogenesis (and probably ovulation) as shown at autopsy in case No. 2 and in the animal experiments. Slight alopecia is unimportant.

Summary

Demecolcin (CIBA Pp. 12 669 A), a new alkaloid isolated by Santavy and Reichstein (1950) from *Colchicine autumnale* shows in animal experiments a toxicity 30 times lower than colchicine and a marked elective depressory effect on granulocytes.

Clinical administration, based upon the favourable experiences so far, shows that Demecolcin can well be recommended for prolonged oral therapy in *chronic myeloid leukæmia* The good tolerance and the lack of toxic side effects with the dosage used here may be stressed. Perhaps it will be possible to influence in a palliative sense certain acute myeloid leukæmias, in a manner similar to the use of Aminopterin (one case reported).

Unfortunately, Demecolcin administered orally or intravenously has not proved a definite therapeutic success in the therapy of other neoplasms (myeloma, lymphosarcoma, Hodgkin's, carcinoma and sarcoma) and cannot be employed in high enough doses because of its leucopenic effect. It may, however, be helpful for injection and external application in the case of some skin tumours.

case of lymphosarcoma produced only equivocal results; and in a case of Hodgkin's disease as well as in two cases of multiple myeloma, it produced no change at all. An attempt to use Demecolcin in lymphatic leukæmias showed that its use here is strictly contra-indicated. In case No. 4, there was an initial improvement, with increased well-being and a drop in the number of lymphocytes (Fig. 5). However, subsequently the granulocyte count dropped even faster, so that for a time an absolute granulocyte count of 74/mm.³ existed. Another patient admitted in an advanced state of subacute lymphatic leukæmia suffered an acute exacerbation and died under the Demecolcin therapy. The terminal lymphocyte count rose from 200,000 to 400,000 with the simultaneous appearance of numerous lymphoblasts. It is difficult to say to what extent the drug was responsible. Similar exacerbations have occurred after X-ray irradiation of these diseases. Since all these cytostatics produce an initial stimulating effect prior to inhibiting the mitotic process, it is possible that that is what occurred here. On the basis of these observations, *we wish to emphasize once again that the use of Demecolcin is strongly contra-indicated in the treatment of lymphatic leukæmia.*

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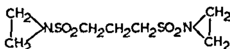
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THE EFFECT OF 1:3-BIS (ETHYLENEIMINO SULPHONYL) PROPANE IN ANIMAL TUMOURS AND HUMAN LEUKÆMIA

EDITH PATERSON and P. B. KUNKLER

THE substance 1 : 3-bis (ethyleneimino sulphonyl) propane, to which I shall refer as B.E.P., was described in 1951 by Hendry, Homer, Rose and Walpole.

1·3-bis(ethyleneimino sulphonyl) propane



IQ694 (B E P)

FIG 1

This sulphonamide is one of a group of substances, examined by these workers, which have two or more ethyleneimine residues in the molecule A common feature of such substances is the possession of marked anti-mitotic and growth inhibiting activity. *Further experimental work on animals with this* member of the group has been carried out by Dr. Walpole of I C I , and he has allowed some of his findings to be quoted

A single dose of 2–4 mg. per kg. given to rats on the day after implantation of the Walker tumour resulted in almost complete suppression of tumour growth for at least fourteen days and this occurred whether the dose was given intraperitoneally or by mouth Visible chromosome damage was present in 90 per cent of the cells examined at anaphase-telophase and bone marrow damage was found.

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DISCUSSION

DANIELLI. If you deacetylate colchicine without replacing the methyl ... in cultures, mitosis ... Schar, Loustalot ... think Dr. Dustin has also worked on it

DUSTIN: If colchicine is deacetylated you get trimethyl-colchicinic acid, whic... tensively ...

About ... chicine af... more than colchicine. It has however a lower toxicity, as evidenced by a much smaller number of nuclear pyknoses in the thymus. However, measures of the eosinophil count in rats indicate that the eosinophils fall there is a positive Thorn test, that is to say a stimulation of the pituitary-adrenal system

extent in the epithelium of kidney and thyroid gland where colloid was scanty. Another dog was treated similarly until death occurred. At post-mortem hæmorrhages were found in the lungs, lymph nodes and gut wall. Microscopically there was clear evidence of damage to the lymph nodes and to the epithelium of kidney and gut.

In the rat the response of the blood is similar to that of the dog, in that neutrophils are depressed while red cells are little changed.

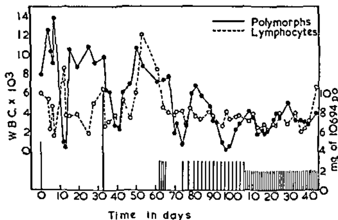


FIG 3 (Same dog as in Fig 2) Effect of B E P. on polymorphs and lymphocytes

The compound has also been tested in myeloblastic chloroleukæmia in the rat. Fig 4 shows clearly the effect on the primitive cells. This rat with a myelocyte count of over 130,000/mm ^{3} would have died in a few days A dose of 15·5 mg /kg. given over twenty-two days clearly lengthened the life of the rat and indeed it is probable that it would have lived longer but for the heavy dosage which was given at the end After the first dose a dramatic fall occurred in the cells of the granular series, particularly in the myelocytes, the lymphocytes fell, but only to their normal range.

The effects on the normal blood count were studied in dogs. Fig. 2 shows some of the effects of B.E.P. given orally to a dog over a total period of five months. The doses shown here include the preliminary sighting doses. Later, doses of 3 mg. at intervals of two to three days were given for a month, and later still doses of 2 mg. were given daily for five weeks. The red cells were hardly affected but the leucocyte count fell

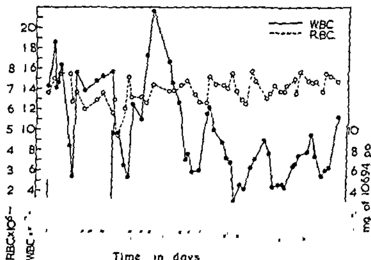


FIG 2 Effect of B E P on peripheral blood in a dog

with each single dose and maintained a lower level during treatment. In Fig 3 is shown the effect on neutrophils compared with lymphocytes in the same dog. In spite of the many fluctuations in the neutrophil count it is apparent that these cells are clearly suppressed while those of the lymphocyte series are less affected. This dog was autopsied two months after the last dose and at this time the neutrophil count had recovered, but the lymphocyte count was about half pre-treatment value. At post-mortem the organs were normal macroscopically but microscopic damage was present in the spleen, the lymph nodes and in the testis, and to a lesser

In only one of these non-leukæmic patients did any improvement in their disease occur. This one example of doubtful benefit was in a case of Hodgkin's disease.

Effects in Leukæmia

We have treated eighteen cases of leukæmia with B.E.P. Two of them were acute, and no improvement was obtained in these acute cases.

Beneficial effects were seen, particularly in the group of chronic myeloid leukæmias. Most of the cases chosen were advanced and most had been treated previously over long periods by more than one method; such methods included X-rays to the spleen, ^{32}P and triethylenemelamine.

Dosage in myeloid and lymphoid leukæmia

The common method of administration of B.E.P. was to give a dose of 0·5–2·0 mg./kg. spread over three days and repeated at approximately monthly intervals or as required. A maximum of eleven such courses have been given with apparent safety. This constituted our maintenance method. In the same cases and in others an alternative method has been used whereby doses were given in small daily amounts over periods lasting for ten to forty days. For the longest period the daily dose was 0·03 mg./kg.; for shorter periods of thirty days or less the daily dose was about 0·15 mg./kg. On the whole this is a less safe method of administration, as overdosage is more likely to occur.

Myeloid leukæmia

The chief application of the drug appears to be in myeloid leukæmia. The criteria of benefit should include an improvement in well-being, which is usually accompanied by a rise in hæmoglobin, and a diminution of an excessively high white count, especially in the number and proportion of immature cells. Some reduction in the size of the spleen is also to be expected.

Effects on Non-leukæmic Blood Count in Man

The effects on the non-leukæmic human subject were examined in ten cases. These patients could not be classed as having a normal hæmatopoietic system as they were either cases of metastasizing solid tumours or advanced reticuloses other than leukæmia.

With very low sighting doses about 1/5 of the therapeutic

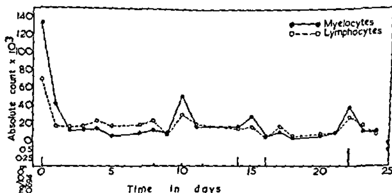


FIG. 4. Effect of B.E.P in myeloblastic chloroleukæmia in the rat Granulocytes not shown.

dose level a slight rise in lymphocytes took place. No change was observed in the hæmoglobin or platelets.

The ultimate dose tested was of the order of 1·0 mg./kg. This was given by mouth over three days. With this dose the neutrophil count fell to about half. The response of the lymphocytes was inconstant, the lymphocytes rose in some, fell in others or remained unchanged. In five cases a decrease in hæmoglobin occurred; this recovered in three to eight weeks. One patient with generalized myelomatosis whose hæmoglobin fell 20 per cent was transfused. Platelets were unchanged in all but two patients in whom a reduction occurred.

The duration of benefit is of importance, and this is less easy to assess. Twelve cases of myeloid leukæmia have been treated; six of these had benefit lasting six months or more. One other is well at three months but is continuing in remission.

Fig. 5 shows a satisfactory response in one of the cases. The reduction in the total white count and in the immature

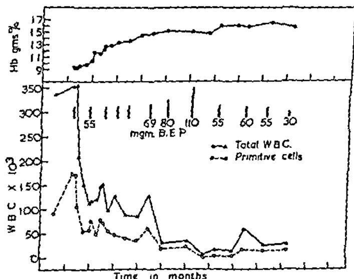


FIG. 5. Response to B.E.P. in a patient with chronic myeloid leukæmia

cells was accompanied by a rise in hæmoglobin. This patient was recently given small daily doses of B.E.P. and has suffered a severe fall of platelets but without showing petechial hæmorrhages. This sequel constitutes an argument against continuous daily dosing.

Responding patients showed an improvement in well-being and this was associated with a rise in hæmoglobin. In the terminal cases the hæmoglobin continued to fall slowly. The total white count was reduced in all 12 patients. The

number of primitive cells and their ratio was reduced in ten cases; two cases, both terminal, showed no reduction in primitive cells. A reduction in the size of the spleen occurred in all but two of the cases. An example of this reduction is shown in Figs. 6 and 7, showing the spleen at the time of treatment and eleven months thereafter.

The effect on the lymphocytes was variable; changes either upwards or downwards were temporary and did not seem to be important. Platelet counts remained within normal limits in the majority of patients. Two patients had an excessively high platelet count on admission; in one of these the count fell to the normal range, in the other it remained high. Thrombopenia occurred in the one patient referred to previously.

Two children with chronic myeloid leukæmia were included in this group It has been our experience that children with chronic leukæmia are rather refractory to treatment, especially to systemic treatment. One of these children showed a poor response to an initial course of B.E.P. (1·0 mg./kg). For this reason splenic irradiation was given and a satisfactory but short remission followed. When the child's hæmoglobin level began to fall again, and the white blood count rose, he was re-treated with B E P. on a daily basis at an average daily dose of 0 18 mg./kg, which is somewhat higher than has been used for adult patients. A good remission followed and the child continues well three months later. A second child who had responded poorly to radioactive phosphorus and to splenic irradiation also showed little benefit from B E P

Four other cases derived no worth-while benefit from B E.P. Three of these patients were clearly in their terminal phase; the fourth was in fairly good condition but had ceased to respond to splenic irradiation or to ^{32}P This patient did not improve with B.E.P. treatment and showed no response to a later course of X-radiation.

Some of our patients have been subsequently treated with either X-rays to the spleen or Myleran for comparison of effects Of four myeloid cases later treated with X-rays only

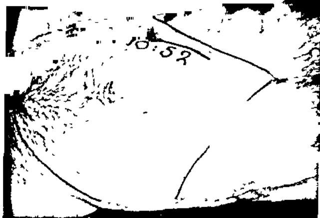


Fig 6

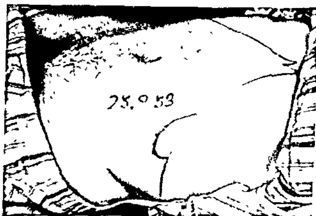


Fig 7

Effect of B E P on spleen size in chronic myeloid leukaemia

Lymphoid leukæmia

Six cases of lymphoid leukæmia were treated, four of them chronic.

The result was disappointing, in that only one case of chronic leukæmia had any real improvement. This patient has remained benefited for a period of over forty weeks. In the three other cases no improvement occurred by any criterion whatever.

Of the remaining two cases, an acute lymphoid leukæmia did not show any real improvement that could not be accounted for by a transfusion which was given at the same time, a subsequent course of B.E.P. without transfusion had no beneficial effect. The remaining case was an atypical leukæmia which did not improve with B.E.P. but which had considerable lasting benefit from the administration of triethylenemelamine.

Side Effects

The side effects of B.E P. include occasional nausea and loss of appetite. This is rarely present in the intermittent method of dosing at 1·0 mg./kg. which covers three days. It may occur with doses of 1·5 or 2·0 mg /kg. Three of our patients have complained of stickiness of the eyes one or two months after starting treatment. This has resolved spontaneously. Two patients have had feelings of tingling or burning in the fingers about one month after commencing treatment. This, too, has been transient.

Summary

The foregoing work demonstrates the effect of a sulphonamide in controlling chronic leukæmia. It is clear that the benefits of B.E.P. are limited to cases of myeloid leukæmia. In this respect it resembles Myleran and differs from triethylenemelamine, which can be employed usefully in either the lymphoid or myeloid types.

The close similarity between the effects of B.E.P. in animals and man is of interest

one showed a somewhat better response to this later treatment. Three were treated with Myleran; the response in these cases was about the same as to B.E.P.

Fig. 8 shows the career of one of these patients. Starting with ^{32}P he had two very satisfactory remissions followed by a third which was only partial. X-radiation to the spleen effected one very good remission. When the white count

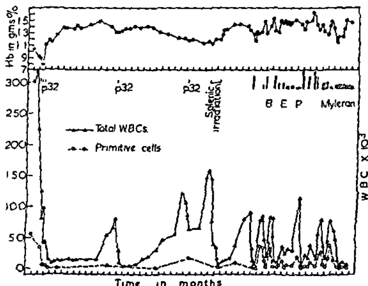


FIG 8. Chronic myeloid leukæmia treated with ^{32}P, splenic irradiation, B.E.P. and Myleran over a period of over four years.

began to climb and the hæmoglobin to fall he was treated with B E P. by an intermittent maintenance method. The hæmoglobin rose, the spleen decreased in size and the white blood count responded intermittently. However he had nausea, so the treatment was changed to Myleran at doses varying between 4 and 10 mg. per day.

At first the patient did not like Myleran, he felt drowsy, the spleen enlarged somewhat and his hæmoglobin was unsteady. Now he is very pleased indeed. His hæmoglobin has risen and he feels well.

while. It is very difficult to shield the kidney and treat the spleen adequately, but I still think that would be one's treatment of choice in the new case.

MOESCHLIN: This is not an answer to Dr. Jacobson's question, but it might interest some of you here: for the new cytostatic drugs we don't know, but we have some data on arsenic (which I still think is a very

with arsenic (Fowler's solution), they have an average life-time of four-and-a-half years Now this is naturally within the limit of error. But perhaps some of these drugs, because they act more selectively on granulocytopoiesis, might bring a prolongation of life for chronic myeloid leukæmia.

thing else.

JACOBSON: Economically and socially it's sometimes very difficult for a patient to be treated with X-rays, and I would like to know when I'm

gives about equally good results But I meant rather that when one is trying any of the newer therapeutic chemical agents, then one should not in the first place use them on fresh cases of myeloid leukæmia with a fairly good prognosis, but the more conservative methods of treatment.

ISRAELS: I should have thought that the more specific the reaction

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DISCUSSION

WITTS. It is striking that we have this rather sharp distinction between the remedies which are effective in acute leukæmia, which seem to be mainly nutritional antagonists, and this group of remedies in chronic leukæmia, whose method of action Prof. Haddow has discussed.

BURCHENAL: There is one compound that does have a little carry-over

studied as long as these other compounds. It has no effect on chronic lymphocytic leukæmia at all.

MOESCHLIN. I was very much interested in the preparation Dr.

Walker tumour in anaphase and telophase twenty-four hours after they had been treated with B.E.P. Presumably this is an expression of damage that probably occurred at the resting stage or in some phase of the resting nucleus

PATERSON. *Yes, one must assume that it happens at the resting phase* unless it happened in the previous prophase and carried on to the following mitosis

WARWICK. The discussion this afternoon raises an interesting point

HADDOW. I think one difficulty is the measurement of clinical improvement, especially in such a short time. There is no question that in various animal leukæmias considerable prolongation of survival can be obtained, and in many ways that is a better measure than any other.

improve the distant bone marrow? There might be much more interesting results than one of our agents which we know has a generalized distribution.

WITTS: The trouble about X-rays is that so often it is a split therapy, where the responsibility for the patient is divided between two people. I think physicians are often biassed towards the drug because they obtain a closer observation. I believe the ideal way is to have a joint clinic, as we have in Oxford, which both the radiotherapist and the hæmatologist attend.

DUSTIN: When we have cases of myeloid leukæmia with high leucocyte counts, we usually treat them with X-rays and urethane at the same

therapist.

WITTS: Another thing is that as a result of the introduction of radioactive isotopes the radiotherapists are becoming more experimentally minded, and are much more co-operative than they used to be.

WARWICK: In our experience there is no method of initial treatment of the disease which is so consistent as irradiation of the spleen. In the

solely.

ISRAELS: All the figures seem to show that whatever you do with myeloid leukæmia today, the average length of survival is the same (although, of course, you have a certain number of people who survive a very short time and a certain number who survive much longer than you expect).

BURCHENAL: I suspect that it makes very little difference what

may leukæmic growth be arrested irreversibly by any chemical acting on mitosis?

New Anti-leukæmic Mitotic Poisons

It is indeed a remarkable fact that most substances tested in recent years on animal or human leukæmia, and used in therapeutics, are mitotic poisons. Their chemical structures are widely diverse, and their action on cell division appears to be the only experimental link between them. The evidence for this anti-mitotic action will be briefly summarized. The drugs belong mainly to the group of "chromosomic" poisons; these may be classified as antimetabolites and antivitamins, alkylating agents, and hormones.

Antimetabolites and antivitamins. The substances which antagonize folic acid—or, more exactly, the active form of this vitamin, the citrovorum factor—and have proved to be of some use in the treatment of human or experimental leukæmias, are all derivatives of 4-amino-pteroylglutamic acid The substitution of an hydroxy- group by an amino- group in position 4 apparently prevents the citrovorum factor from acting as a one-carbon donor, one of its main physiological functions Folic acid deficiency, either nutritional or the consequence of antimetabolite action, results in cellular destruction in bone marrow and intestinal mucosa (Philips and Thiersch, 1949). The epithelial cells of the intestinal crypts of the mouse provide an excellent material for the experimental study of mitosis After injections of several antifolic drugs (Aminopterin, Amethopterin, Amino-teropterin, Aminopterin, Adenopterin), the following changes have been described in these cells (Dustin, 1950*b*): mitotic arrest (preprophasic inhibition), pyxnonecrotic nuclear destruction, nuclear swelling, and later, resumed mitotic activity with abnormal mitoses demonstrating chromosome breakage. Such changes parallel closely those observed in the same tissue following irradiation, and, from this point of view at least, *the antifolic drugs are "radiomimetic" or "chromosomic" mitotic poisons.*

THE GROWTH-INHIBITING ACTION OF MITOTIC POISONS IN EXPERIMENTAL AND HUMAN LEUKÆMIAS

P. DUSTIN, Jr.

MOST substances which have proved of some utility in the chemotherapy of malignant growth are mitotic poisons, i.e. substances particularly toxic for dividing cells. This was discussed by the author at the 1947 Cancer Congress, where reference was specially made to the following drugs: nitrogen-mustards, acridine derivatives, carbamates, quinones, arsenical derivatives, colchicine, bacterial polysaccharides and heavy metals (Dustin, 1949). Hormones were not discussed, for too little was known of their action on the cellular divisions of malignant growths.

In the last five years, many other chemotherapeutic drugs have been discovered (Skipper, 1953). The most important have proved useful in the treatment of some forms of leukæmia. Most of these chemicals appear to be endowed with some type of toxicity towards dividing cells, which they alter either before prophase by disturbing the formation of new chromosomes (chromosomic or "radiomimetic" drugs), or at metaphase by destroying the spindle (spindle poisons).

In 1947, it was pointed out that the growth-inhibiting properties of such substances could be explained either by their action on mitosis alone, or by some action "beyond mitosis" on a fundamental biochemical mechanism of the malignant cells or of the hypothetical agent of malignancy. The purpose of the present paper is to examine anew these hypotheses, after giving a summary of what is known about the mitotic-poisoning action of the chemotherapeutic agents studied since 1947. It is hoped that this may clarify the present problems, and provide a tentative answer to the question:

against some malignant growths and some types of lymphoid leukæmia in mice (Law, 1950), induces considerable nuclear changes and inhibits the growth of some tumours (Woodside *et al.*, 1953) However, the doses which are effective against neoplastic growth do not affect the normal mitoses, as demonstrated by the colchicine method (Woodside *et al*, 1953). The nuclear abnormalities observed in sensitive tumours suggest that the action is mainly a "chromosomic" one, which is not surprising considering the results obtained with diaminopurine.

Another antimetabolite which may be mentioned here is desoxypyridoxine, an antagonist of pyridoxine capable of inducing in the rat signs of specific vitamin deficiency This substance has been reported to inhibit the growth of lymphosarcomas (Stoerk, 1950), but its action on mitosis does not appear to have been analysed

Alkylating agents. These cytotoxic drugs belong to several different chemical groups, but have in common the property of combining very readily at low temperature with many important body constituents, in particular with the nucleic acids (Ross, 1953) The best known is nitrogen mustard (chloroethylamine). This was demonstrated many years ago to be a typical radiomimetic mitotic poison (Dustin, 1949, 1951*a*). It is the most "radiomimetic" of all the drugs of this type, being at the same time a mitotic poison for all types of mitoses, a mutagen and a carcinogen Other chemicals may be "radiomimetic" for one or some types of tissues only, e g the antifolics, which arrest mitoses in intestine and bone marrow while not affecting the lymphoid tissues. One of the most widely used of the "new" alkylating agents is TEM (triethylenemelamine) its cytological action is similar to that of the chlorethylamines, a fact which becomes clear when it is recalled that these are rapidly hydrolysed in the body, with formation of the reactive imonium ion, which has an ethyleneimine structure.

The most interesting of the alkylating agents, because it has rapidly gained a wide use in the treatment of human leukæmia

The antimetabolite, diaminopurine, which is a *guanine* and (or) an adenine antagonist, induces exactly the same changes in this tissue (Dustin, 1950*b*) (cf. Philips and Thiersch, 1949). Biesele *et al.* (1952) have recently confirmed its chromosome-breaking properties. Although this substance has only been of limited use in therapeutics,* in a leukæmic strain of mice Burchenal *et al.* (1949) have obtained a prolongation of life as great as after folic acid antagonists. The figures are: survival time of controls after grafting, 19·5 days; methyl-*bis*-β-chloroethylamine: 24·8 days; diaminopurine: 31·9 days; Amethopterin: 32·6 days.

Both the antifolic drugs and this purine antagonist induce strong degenerative changes in the bone-marrow, eventually leading to aplasia. While the relation of these changes to mitotic inhibition is more difficult to analyse than in the intestine, all the experimental data suggest that mitotic inhibition of the "radiomimetic" type plays the greatest part. In such action mature non-dividing cells are not affected by the poison, a fact which is confirmed by all work on leukæmic leucocytes.

It should be mentioned that some authors, for instance Jacobson and Webb (1952), consider Aminopterin a spindle poison, for in tissue cultures of fibroblasts they observed a typical metaphase inhibition of mitosis. However, the concentrations of Aminopterin (1 : 2000) are far greater than those which are effective in leukæmia, and in mice it has not been possible to observe any evidence of metaphasic arrest or spindle-changes in animals injected with 1 mg. (±50 mg./kg.) and observed during the first sixty minutes after the injection. The only change was a decrease in the number of mitoses, with no modification of the metaphase-anaphase ratio (P. Dustin, unpublished)

One of the most remarkable antimetabolites is azaguanine (guanazolo). This antagonist of *guanine*, which is effective

*Another purine antagonist, 6-mercaptopurine, has been reported to be effective in human leukæmia (Burchenal *et al.*, 1953). No cytological data are yet available.

of lethal doses of Aminopterin, for instance, that nuclear destruction is observed in the thymus and lymph glands, and this is probably only a secondary and non-specific consequence of a general toxic action.

It is evident that if adrenal steroids are mitotic inhibitors, any adrenal stimulation will affect mitosis, and that ACTH, and all actions stimulating the release of ACTH, will indirectly affect mitosis. These facts suggest an explanation of the "choc caryoclasique" studied by A. P. Dustin since 1925, and of the antileukæmic actions of these hormones.

Spindle poisons. These drugs interfere with cell-growth by preventing the spindle from pulling the chromosomes apart at anaphase. From the extensive researches on colchicine it appears that the principal effect is a progressive loss of the fibrous and polarized structure of the spindle, which changes into an amorphous mass, which has been called either "pseudo-spindle" or "hyaline globule" (Gaulden and Carlson, 1951). Chromosomes are only affected when the cells undergo destruction after their mitoses have been arrested for many hours at metaphase

The effects of colchicine need not be considered here. methyl colchicine, which has been discussed by Prof. Moeschlin, has a similar spindle effect but its toxicity is lower (P. Dustin, unpublished). While many substances are known to affect similarly the spindle in plant cells, the number of spindle poisons in animals is quite limited, and several have only been tested in tissue-culture work Podophyllotoxin, which has been used in human therapeutics against skin papillomas, has been shown to slow the growth of animal leukæmias (Waterman, 1950). Its action on cell-division is similar to that of colchicine.

On the other hand, mention should be made of the oldest of all anti-leukæmic drugs, sodium arsenite: this, and several arsenical derivatives, such as dimethyl-arsinate (cacodylate), are typical spindle-poisons, as demonstrated in mice by Piton in 1929, and by Limarzi's observations (1943) of bone-marrow in pernicious anæmia

is dimethane-sulphonoxy-butane ("Myleran"). This is also an alkylating agent, but no detailed information has yet been published about its action on mitosis. The fact that it inhibits the growth of cells of the myeloid series both in the rat and in man is indirect evidence of some action on mitosis. This substance, being mainly soluble in lipids and very slightly in water, acts more slowly than water-soluble substances like TEM.

Hormones. Many hormones promote mitosis, e.g. the gonadotrophins, prolactin, the thyrotrophic fraction of the anterior pituitary, œstrogens and testosterone. While this action is mainly visible in one or two specific receptors, Bullough (1946) has demonstrated that œstrogens stimulate cell-division in many tissues. The existence of hormonal antagonisms suggests that a depression of mitoses may be observed, for instance in genital tissues, after injection of the steroids belonging to the other sex. It has long been known that the "karyoclasic" destruction of the cortical cells of the thymus is identical with that brought about by the direct action of radiation, or toxic substances (e.g. colchicine, Leblond and Segal, 1938) injected directly into the gland. When it was demonstrated that the thymic changes of the "alarm reaction" were the consequence of adrenal stimulation (Selye, 1946), it could be suggested that cortisone and (or) other steroids were the "endogenous mitotic poisons" the existence of which had been suggested in 1946 (Dustin).

The careful analysis of mitotic rate in epidermis by Bullough (1952) has provided clear evidence that cortisone and probably other closely related steroids are capable of decreasing the numbers of new mitoses, and thus have an action comparable to that of the weak chromosomic mitotic poisons. In recent

some cells and tissues is paralleled by that of the antifolic drugs, the main targets of which are the intestinal mucosa and the bone-marrow (Dustin, 1951*a*, *b*). It is only after injections

(hæmocytoblastic) leukæmia. Cultivation of the bone-marrow *in vitro* in the presence of colchicine enabled these authors to rule out the possibility that the low mitotic index may be the consequence of abnormally rapid mitoses of the malignant cells. In fact, the growth of hæmocytoblasts in leukæmia does not appear to be quantitatively more important than that of normal bone-marrow, and may even be less, a fact which agrees with many observations of bone-marrow aplasia linked with an incipient leukæmia. The absence of cellular maturation is the main reason why the abnormal cells pile up in the bone-marrow and the hæmopoietic organs, and eventually invade the blood-stream.

This point should be kept in mind, and applies to any type of malignant growth, as emphasized by Kidd in 1946: the principal characteristic of malignant cells is not their rate of growth, but their abnormal or incomplete maturation and differentiation. Leukæmias, alone of malignant tumours, have the property of continuously shedding the newly-formed cells into the blood-stream, where they only survive a relatively short time This fact makes clear at once why myeloid leukæmias, with short-lived differentiated cells, are the most amenable to any type of therapy influencing cellular multiplication Crypto-leukæmias and the closely related sarcomas, the cells of which do not move into the blood, are much less influenced by therapy, unless this destroys the cells locally (e g. radiation)

Another point related to mitotic growth in the bone-marrow should be mentioned. It is a commonplace observation that any treatment of leukæmic growth will improve the numbers of red blood cells Before discussing whether this means that erythroblastic mitoses are more resistant than leucocytic ones, which seems a logical conclusion, an important observation must be made. It appears clear, as mentioned above, that DNA synthesis and leucocyte mitosis go hand in hand, and that a complete set of chromosomes is duplicated at each division (or, more correctly, before each division). It is not so evident that the same holds true for erythroblasts,

Mitotic Growth in Leukæmia

Most chemotherapeutic drugs which have proved of some use in malignant disease in man have been found effective against leukæmia. This is evidently not only because leukæmia, with the possibility of quantitative assessment of growth, is better fitted for testing new drugs. It is more probably the consequence of the conditions of growth of the leukæmic cells; and before discussing the relations between mitotic poisons and leukæmia, some aspects of the growth of malignant white blood cells should be considered.

Quantitative data about the mitotic growth and life-span of malignant leucocytes are scarce, but it must be kept in mind that there is no strong evidence that growth of malignant cells is much faster than that of normal leucocytes. Because of the short duration of the life of the granulocytes, which does not exceed a few days, the "turn-over" of these cells is very rapid. In man, about three-quarters of the bone-marrow is active in replacing the leucocytes, which from a quantitative point of view are only a minor constituent of blood. In chronic myeloid leukæmia, a similar evolution of white cells takes place. Osgood *et al.* (1952) calculated the life-span of leucocytes in various types of leukæmia from a study of the deoxyribonucleic acid (DNA) incorporation in the newly-formed cells (DNA synthesis takes place in close relation to mitosis, and provides a rather reliable index of mitotic growth). They found that in chronic myeloid leukæmia and subacute monocytic leukæmia the survival time of the newly-formed leucocytes was three days, that is to say about normal In chronic lymphatic leukæmia, on the contrary, the abnormal cells lived as long as one hundred days, a fact which may explain the resistance of this type towards chemotherapy. In "acute" leukæmias of the lymphoid type, life-span of the abnormal cells was less than three days.

This point deserves some comment, for Astaldi and Mauri (1953) have quite recently pointed out the low mitotic indexes observed in the bone-marrow of patients with stem-cell

man. Even if they act by depressing the mitoses of the immature cells, they do so because these are only possible when high concentrations of folic acid (probably converted to citrovorum factor) are present.

The antimetabolites interfering with purine metabolism may act similarly. It is well known that purine molecules are utilized in the synthesis of nucleic acids, and that cellular requirements differ considerably in this respect. Malignant cells which are unable to synthesize adenine and (or) guanine will be influenced by substances such as diaminopurine or 6-mercaptopurine. Even if it is demonstrated that the result of this therapy is a change in the mitotic rate of the cells, this is evidently only a consequence of their particular nutritional requirements. Azaguanine ("guanazolo") is the most interesting of such drugs, not so much from a practical point of view, but because of the resistance of normal mitoses to its action (Woodside *et al.*, 1953). So far, this is the only chemotherapeutic drug effective against some forms of acute lymphoid leukæmias in mice which affects neither intestinal nor lymphoid mitoses. Law (1950) found that the leukæmic cells require a more abundant supply of guanine. Resistant cells have been demonstrated to be able to detoxify azaguanine to 8-azaxanthine by deamination (Gellhorn, 1953). Here again, if mitotic changes were observed in these cells, they would only be the *consequence* of a particular metabolism, i.e. absence of azaguanine deamination.

Another instance which certainly deserves further study is the action of benzene in human myeloid leukæmia Braier (1953) claims that, in the favourable cases, the results are as good as those obtained by radiotherapy, and that the general toxicity can be controlled by the administration of methyl group donor[illegible] mitosis in [illegible] which is one [illegible] powerful mitotic poison (Dustin, 1950*a*, Parmentier, 1952).

In the literature on the chemotherapy of experimental leukæmias, many data are to be found which point towards a

especially during the divisions of the most mature ones. Microphotometric measurements by Marinone (1951) and Korson (1951) indicate a progressive decrease of the DNA in erythroblastic nuclei. While it is evident that mitosis in the youngest cells of the erythroblastic series must involve a complete duplication of all cellular structures, including the chromosomes, it is not so clear whether this is necessary in the later stages, when the nuclei are reaching the time when they will completely disappear. Mitosis without DNA synthesis may be possible, the chromosomes being simply segregated into two genetically unequal groups. This remains to be proved, but as it has been shown that most mitotic poisons disturb mainly the synthesis of DNA (Dustin, 1952), such mitoses without synthesis would naturally be much more resistant.*

Chemotherapeutic Effects Unrelated to Mitotic Poisoning

While most anti-leukæmic substances have been proved to interfere with mitosis, this has only been observed, for most of them, under experimental conditions, and often with fairly large doses. Some evidence which cannot be neglected points towards other types of action, related to particular metabolic requirements of malignant leucocytes. On the other hand, there are many facts which indicate some specific action of drugs towards particular types of cells, which are difficult to understand if mitosis alone were affected

The observations of Swenseid, Bethell and Bird (1951) that human leucocytes contain more folic acid in leukæmia, and that this increases when the cells are less differentiated, appear to explain the action of antifolic drugs. These substances have never been proved to be of any use in chronic myeloid leukæmia and may be harmful in chronic lymphoid leukæmia in

biological differences exist between the two main types of bone-marrow cells. One important, if indirect, proof of this is the extreme rarity of true malignant growth of the red blood cells in mammals (malignant erythroblastosis, Di Guglielmo's disease).

The Relation of Chemotherapeutic Action to Mitotic Inhibition

Although there are many facts which demonstrate different types of action of the various anti-leukæmic drugs, there remains strong evidence that their action is mainly related to the mitotic-inhibiting properties described in the first part of this paper.

The most striking fact is that substances of very different chemical constitution, acting on cell-division either by disturbing the formation of new chromosomes or by preventing the normal function of the spindle, have similar anti-leukæmic effects. Between podophyllotoxin and cortisone, for instance, there is only one property in common both decrease the numbers of cell divisions, although by considerably different mechanisms.

That chemotherapeutic agents do depress mitotic activity in leukæmia is, however, more than a speculation based on indirect experimental evidence In cases of human acute leukæmia treated by antifolic substances, the numbers of dividing hæmocytoblasts are observed to decrease (personal observations). Moeschlin (1948) demonstrated that urethane (ethylcarbamate) also decreased the number of mitotic cells in human leukæmias. Cramer (1952), studying the action of cortisone in lymphoid leukæmias of children, reaches the same conclusion, that there is a "selective inhibition of mitosis of the leukæmic cells . . . during the first days of the treatment" This appears to him to be "the only criterion for the reactivity to cortisone"

While the existence of some degree of "specificity" is exemplified by the action of cortisone on lymphocytes, of antifolic substances in acute leukæmias, of "Myleran" in

"specific" action of some poisons on some types of leukæmia, or a "specific" sensitivity of some strains of leukæmic cells. The basis of such "specificity" is not known, and will not be discussed here. Some of the most salient facts will be quoted, and should serve as a basis for further discussion.

In acute leukæmias, it is well known that resistance to antifolic drugs and to cortisone may develop during the treatment: the fact that cells resistant to one drug remain sensitive to the other points to a more complex action than a simple inhibition of mitosis (Bernard and Mathé, 1953). Kirschbaum *et al.* (1950) have studied several types of leukæmias in the F strain of mice. In four lines of myeloid leukæmia, Aminopterin and Amethopterin were less effective than potassium arsenite and ethylcarbamate. The opposite was true in certain other lines of transplanted lymphoid leukæmias. In a line of myeloid leukæmia in mice, Engstrom *et al.* (1947) obtained positive results with ethylcarbamate, while benzene, Fowler's solution and colchicine were without effect. Law obtained a "definite, regular and reproducible inhibition" of two transplantable acute lymphoid leukæmias in mice with azaguanine. This substance had no action on a transplantable lymphosarcoma, and other authors, using different strains of leukæmia cells, have found no inhibition of growth. Fowl leukosis was favourably influenced by large doses of antifolic drugs, while nitrogen mustard and ethylcarbamate were without action (Bessis *et al.*, 1951). The same "specificity" applies to hormones like cortisone. In a series of experiments, Sugiura *et al.* (1950) inhibited the growth of one type of sarcoma in the rat (R 39) while sarcoma 180 was unaffected.

The apparent resistance of the erythroblastic cells may also be explained in similar terms of "specific" action (Kindred, 1947). The less specific of the mitotic poisons, for instance the mustards and ethyleneimines, are the most harmful for erythropoiesis, contrary to cortisone and the antifolics. While this may be linked with the particular type of DNA metabolism of the erythroblasts, it is evident that many other

appears to be a simple antagonism of the metabolism of adenine, guanine and also hypoxanthine. Morgan (1952) has extensively studied factors inhibiting the growth of psittacosis virus, and found that antifolic substances, diaminopurine and azaguanine had an inhibiting action. Jacobson, *et al.* (1952) found 2 : 6-diaminopurine to have a destructive effect on the kappa factor in *Paramecium*. This factor is closely related to viruses. On the other hand, though the growth of the Rous sarcoma in chicks may be inhibited by folic acid deficiency (Woll *et al.*, 1951), Ringsted (1952) reached the conclusion that it was only the incubation period which was affected by aminopterin, and that the rate of growth of the tumour was not modified. Evidently no conclusions can yet be reached, since this problem is closely linked with the complex and difficult one of antiviral therapy

One fundamental aspect of the treatment of leukæmia has been left out of the picture in this paper· that of cellular differentiation. The reason is that in our opinion no experimental evidence, and no observations in patients, prove that chemotherapy may improve the abnormal differentiation which is the cause of malignancy When, in the course of chemotherapy, more mature leucocytes are seen to replace the immature ones in the bone-marrow and in the blood, or an erythroblastic proliferation that of malignant hæmocytoblasts, the explanation is that several lines of cells persist Any more or less specific depression of the growth of the abnormal ones will favour the multiplication of the remaining normal leucocytes and erythroblasts

It will be objected that in chronic myeloid leukæmia, cellular differentiation appears to improve under the effect of therapy, whether by X-rays or chemicals. This may also be explained by the co-existence of normal and neoplastic cells, and no proof of a direct effect on differentiation appears to have been given so far. It is our belief that future research, without neglecting the study of more specific antimetabolites and the problems of virus chemotherapy, should aim at a better explanation of one of the most fundamental problems

chronic myeloid leukæmias, and by the existence of resistant strains of leukæmic cells towards some of these agents in animals (Burchenal *et al.*, 1951; Hirschberg *et al.*, 1952; Law and Boyle, 1951; Skipper and Burchenal, 1951), *a non-specific action on cell-division appears to be the main explanation of the therapeutic results obtained to date.* As Geisse and Kirschbaum wrote in 1950, "most of the agents which inhibit the development of transplanted leukæmia also depress body growth, indicating that their effect is not specific for cancerous tissue."

Such a non-specific action clearly explains, considering the growth properties of human chronic myeloid leukæmia, why this has proved to be the best test-object for many anti-leukæmic drugs. The most modern of these are only capable of producing results similar to those obtained with Fowler's solution in the last century. The most promising drugs are the antimetabolites, for here the therapeutic action is only related to mitosis in an indirect way, the metabolism of the malignant cells playing a fundamental part.

Conclusions

The following points appear to need no further discussion here: (1) antileukæmic agents, old and new, interfere *in some way with mitotic growth, and this explains much of* their action; (2) evidence of more specific activities towards some strains of leukæmia, or different types of malignant leucocytes, cannot be denied; (3) the favourable results obtained in leukæmia by chemotherapy, as compared with other malignant growth (for instance, epithelial tumours) is a consequence of the short life-span of these neoplastic cells.

Are there any new facts which would indicate that mitotic poisons are capable of reaching "beyond mitosis" (Dustin, 1949), acting possibly on the causal agent of leukæmia? Some results with different strains of viruses may be mentioned here Matthews (1952) found that the virus of luzern mosaic, when grown on *Nicotiana*, was inhibited by azaguanine. This

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DISCUSSION

in biology and pathology: cellular differentiation and its causal analysis.

Summary

Nearly all substances which have been used with good results in experimental and human leukæmias inhibit mitotic growth, either by disturbing the mechanism of chromosome reduplication (chromosome poisons) or by preventing the spindle from functioning at metaphase (spindle poisons).

The significance of this action on mitosis is discussed, and the importance of more specific actions on some types of cells, or on malignant versus normal cells, is discussed.

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ISRAELS. In other words, if you see a lot of mitoses, your case is probably resistant. I agree.

POLLI. I think it might be valuable to consider some lines of research which have been applied to other biological problems but not to leukæmic studies. It is rather difficult to understand why there have been so few cytological studies on isolated components in normal and leukæmic cells (for instance on mitochondria, microsomes, and chromosomes)

Since 1944, in collaboration with the Genetics Institute and the Zoological Institute, we have studied both isolated chromosomes in the resting stage and mitotic nuclei, in normal and leukæmic cells. With

deoxyribonucleic acids At this time studies are being carried on to see if some of these biochemical features characterize leukæmic nuclei.

WITTS Do you agree with this view that the mitoses are less frequent in acute leukæmia?

POLLI I have never studied the curve of mitotic activity, but I think that mathematically one can object to these counts

MOESCHLIN· In some cases of malignant forms we have found increases of two to three times in the mitotic index in chronic myeloid leukæmia But I think the essential point is that the normal bone marrow has a very variable mitotic activity A pneumonia patient will have an

sometimes in the lymph glands, so there is an enormous increase of active tissue Even if you only have a normal mitotic activity, the total number of mitoses is enormously increased

Moreover, there need not be an increased destruction of cells, as Biermann, Lanmann (*Blood*, 1951, 6, 770) and many others have suggested

rational to attack the question of differentiation than the question of mitosis. I would like to hear some opinions on that point.

HADDOW: We are coming to feel rather strongly that the primary effect is one involving a loss of differentiation, which automatically brings about an enhanced rate of division. As J. A. Murray used to say malignant cells have not *acquired* the power to divide—as normal cells they

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carcinoma, which is not malignant, doesn't differentiate at all.

HADDOW: I agree, the relation is not quite perfect; and this small fraction proves that it is not the only factor.

GORER: Also, even in the normal body there are undifferentiated cells which aren't dividing very much, as far as we know.

ENGELBRETH-HOLM: Of course, we can't generalize. The different tissues must be considered separately.

DUSTIN: I'm afraid I didn't make one point quite clear. Of course, in pathology we all know of very differentiated tumours which are very malignant, so it is not a general rule. But in leukæmia it has particular importance, because when a cell differentiates into a poly-

leukæmia so well is that we have to give something that will actually destroy the cells, whereas in chronic myeloid leukæmia if you stop the

in the bone marrow. But in the normal bone marrow these 6/1000 mitoses will give cells which will eventually become red cells and polymorphonuclears; here they always go on giving the same cells. There is

THE DESIGNING OF SELECTIVE DRUGS

J. F. DANIELLI

Introduction

My approach to this subject will be based upon the thesis that there are many biological variables concerned in determining the selectivity of a drug, and that these variables must be successfully exploited to obtain the highest possible degree of selectivity. As I have shown in a detailed discussion elsewhere, therapeutic efficiency in a series of drugs is limited by different biological variables with different drugs (Danielli, 1950) Consequently it is often impossible to design a sensible scientifically planned investigation unless adequate knowledge is available of the biological variables which may become decisive Conversely, if a programme is planned to exploit biological variables in addition to physicochemical variables, there must be a correspondingly higher chance of success.

Generally speaking, one may endeavour to control or eradicate a tumour chemically either by use of a natural hormone or of a substance of analogous action (e.g. œstrogens), or by use of a toxic substance (e g β-chlorethyl compounds, antimetabolites, and radio-isotopes). In both cases utility of the substance is limited by its action upon cells other than those of the tumour. We are thus faced with the problem of finding means whereby the action of a substance may be limited to narrower ranges of cell types. How is this to be done? So far as I am aware, the only general approach to this problem yet set out is that of making the action of a drug depend upon the simultaneous exploitation of a number of biological variables (Danielli, 1952). If the action of a drug depends on only one biological variable (e g. specific protein synthesis, in the case of nitrogen mustards) its action cannot be highly selective The reason for this is that any single variable is likely to be of great importance for a wide variety

mature, but when myeloid cells are grafted into the subcutaneous tissue or muscle, the tumour cell population there may be composed of myeloblasts maturing only as far as promyelocytes. If this population indicates the range of the leukæmic cells, then out of 200,000 in the blood, not more than, perhaps, 10,000 are leukæmic cells. It follows that the simple mitotic index of bone marrow is of little value. We need to learn how to distinguish between the immature normal cells and the leukæmic cells.

DUSTIN: The work on the mitotic index in bone marrow that I mentioned was only in acute leukæmia. In chronic leukæmia no study of that kind has been done as far as I know. The low apparent number of divisions in acute leukæmia seems to have some relation to the fact that so many acute leukæmias in man begin with an aplastic condition

FURTH: That's the best situation to study.

aff

tha

will have an action upon a cell or not, in practice a second family of variables is also important since it determines the concentration of a drug to which the cell can be exposed. These variables include urinary excretion, detoxication mechanisms and central and other side effects.

Here I shall consider (a) enzymic activation of drugs; (b) enzymic inactivation of drugs; (c) permeation, facilitated diffusion and active transfer in relation to drug design; (d) the nature of adaptation and resistance to drugs; (e) the exploitation of adaptation and resistance phenomena. The discussion will be in terms of nitrogen mustards, but a very similar approach can be made to the problem of securing selective action of growth control substances, antimetabolites and radioisotopes, and we are in fact commencing a preliminary experimental study of œstrogens along similar lines.

Enzymic activation of drugs

The principle in some preliminary experiments which I shall consider is that of choosing an active nitrogen mustard which can be administered as an inert derivative from which the active molecule is likely to be liberated by enzymic action in the body. One of my colleagues, Mr. P Hebborn, has carried out the experiments which will be described here. The drug chosen was *A*, the nitrogen mustard derived from *p*-phenylenediamine. This mustard has a high toxicity, but it seemed probable on theoretical grounds that if the free amino group were combined in a peptide or some similar linkage an inert compound would be derived, since in such a molecule the rate of ionization of the chloride ion should be greatly diminished Other studies, particularly those by Ross at the Chester Beatty Research Institute, had provided the information upon which this prediction could be made. It is to be expected that such a compound would be relatively inactive in the body, but if at any particular site an enzyme were available which would release the parent substance there would immediately ensue a drastic local action. By so reducing the activity of the parent drug we are in fact endowing

of cells. If the action of a drug depends upon two biological variables its action will be more selective than if it depends upon one, since any two variables will be very important to a more limited range of cells than either variable taken singly. For example, a drug which acts upon protein synthesis will act upon the formation of erythrocytes, leucocytes, platelets and intestinal epithelium in particular, and probably will act upon the liver also. A drug whose action is dependent on a high concentration of alkaline phosphatase will exert marked effects on the kidney proximal tubules, intestinal epithelium, choroid plexus and regenerating or calcifying bone. But if the action of a drug upon protein synthesis depends upon the presence of a high concentration of alkaline phosphatase, we should expect its action to be largely restricted to the intestinal epithelium; and in general it can be asserted that the larger the number of biological variables that condition the action of a drug, the more highly selective will that drug be.

Among the variables which might be exploited are included enzymic constitution, permeability properties, facilitated diffusion, active transfer, chromosome breakage, protein synthesis, nucleic acid synthesis, gene reproduction, adaptation to drugs, resistance to drugs, etc. We know a good deal about the enzymic constitution of different cells. The extent of our theoretical knowledge about permeability by thermal agitation is satisfactory, but there is little detailed information available in which the permeability of cells of different lineages has been compared. We are beginning to develop the theory of facilitated diffusion Of the theory of active transfer we are still almost completely ignorant. We are equally ignorant of the mechanism of chromosome breakage, protein synthesis, nucleic acid synthesis, adaptation to drugs and resistance to drugs. Thus the fields which can be exploited are limited to enzymic constitution and permeability properties and certain small areas of knowledge in connection with the other variables.

In addition to the types of variables mentioned above which are primarily concerned in determining whether a drug

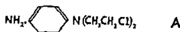
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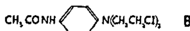
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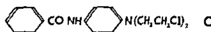
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the nitrogen mustard with the equivalent of a proximity fuse, which will be touched off only by a limited range of enzymes. Although it is possible that the compounds formed by inactivating *A* through formation of a peptide linkage with an amino-acid would ultimately be of most interest, the difficulties in synthesizing such compounds have led us to make our initial experiments on the more readily available acetyl and benzoyl compounds *B* and *C*. Both the parent substances and these two derivatives were kindly prepared for us by Mr. D. A. Firth of Boots Pure Drug Co. Ltd.







The action of the drugs was studied upon the Walker rat carcinoma 256 growing subcutaneously. We are indebted to Mr. Marsh of the Chester Beatty Research Institute for providing the tumour and for help in the initial stages of the work. The approximate LD_{50} for these different compounds were as follows:—

Amino compound .	0 8 mg. per 100-g. rat
Acetyl compound . .	4 8 mg. per 100-g. rat
Benzoyl compound .	35 mg per 100-g. rat

From the above figures it is clear that formation of the *N*-acetyl derivative has had the predicted effect of greatly reducing the toxicity The drugs were then compared as therapeutic agents by using them to study well established tumours, *administering a daily dose of 1/3 of the LD_{50} for a* number of days. The drug was never administered until the tumour had become well established, was of measureable size

(about 5 g.) and was growing rapidly. Administration of the drugs often caused a considerable loss in weight, and injections were discontinued if the weight loss showed any sign of exceeding 15 per cent. In animals treated with the free amino compound, the rate of growth of the tumour was slowed down and there was a substantial loss in weight of the animal. When the administration of the drug was discontinued the animal would increase in weight again, but the tumour also resumed its rapid rate of growth. In the case of the acetyl compound, there was a similar loss in weight of the animal but the tumour, instead of growing, diminished in volume and its rate of growth was commonly slowed down for a considerable period after administration of the drug had been stopped. But in the majority of cases the tumour eventually again began to grow, and when this occurred it was usually relatively resistant to the action of the acetyl compound, so that if a fresh course of treatment with the acetyl compound was given, although the acetyl compound again produced a decline in weight of the animal, it would no longer produce a decline in weight of the tumour. From these results it was clear that acetylation, as was hoped, had resulted in a much greater degree of selectivity of the drug for the tumour, so that the prediction of a given degree of toxicity to the animal as a whole produced much more damage to the tumour in the case of the acetyl compound than in the case of the parent compound. But, on the other hand, the tumour was rarely completely eradicated and when growth was resumed a resistant condition was evident.

The benzoyl compound was still less toxic, but had little action upon either the weight of the animal or the size of the tumour, even when given in a dose sufficient to cause death of the animal. It was evident from the condition of an animal killed by the action of the benzoyl compound that it produces death by a different mechanism to the parent substance and to the acetyl compound. It might in fact be said that benzoylation has produced a substance which no longer has a direct action upon those cells, including tumour cells, which

are the most readily attacked by the parent compound. Death caused by the benzoyl compound is due to a rather highly selective action upon a tissue so far unidentified.

The question now arises as to whether the results obtained from these three drugs are really to be explained in terms of enzymic activation of the acetyl compound and failure to activate the benzoyl compound. To obtain evidence upon these points, we investigated the enzymic content of typical tumours. The tumour was found to contain an enzyme which would liberate the parent substance from its acetyl derivative but not from the benzoyl derivative. Thus the evidence from enzymic studies is compatible with the view that enzymic activation really is responsible for the increased therapeutic selectivity of the acetyl as compared with the amino compound

Selectivity based on inactivation

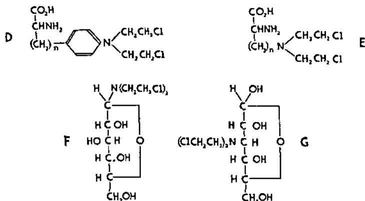
If an enzyme is ubiquitous in its distribution, then in theory it is possible to obtain a selective attack on a cell type which lacks this enzyme, by using a drug which will be inactivated by the enzyme. A number of enzymes show this type of distribution, *e.g.* triose phosphate dehydrogenase and the cytochrome system, but so far no drugs have been designed to this pattern, though Dr W. C. J. Ross tells me that he has the matter under consideration.

Selectivity based on Permeability, Facilitated Diffusion or Active Transport

By *permeability* I refer to the ability to diffuse into cells by thermal agitation. Although differences in permeability between cells are known to exist of a magnitude which would be significant in considering drug design (Danielli, 1950), too little is known of the permeability of mammalian tissue and tumour cells to make this approach profitable at the moment.

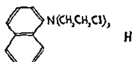
By *facilitated diffusion* I refer to the penetration of cell membranes through patches on the membranes which selectively facilitate the passage of certain molecules, without the

expenditure of energy. Facilitated diffusion is usually limited to physiologically important molecules such as sugars and amino-acids. Variations from cell type to cell type are very striking For example, glucose permeates sheep red cells by diffusion, and human red cells by facilitated diffusion. In given circumstances it takes about 1000 times longer for a given amount of glucose to penetrate into sheep red cells than into human red cells. Although very little is known about facilitated diffusion in tissue cells, the order of magnitude of the variation in this variable from cell to cell is so large that it might nevertheless be profitable to consider a few drugs which might become selective through facilitated diffusion Examples of such compounds are the compounds containing a mustard grouping allied to a sugar or an amino-acid. For example, the compounds *D*, *E*, *F* and *G* would be of interest.



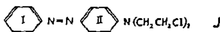
So little is known of the mechanisms of active transport that exploitation of this variable must remain difficult for some years. But active transport is very selective. For example, in the absence of fat-mobilizing hormones the adipose tissue cells far exceed any other cell type in the efficiency with which they pick up colloidal fat from the blood. Thus a tumour of fat cells or a tumour which readily accumulates

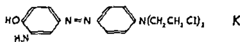
colloidal fat might well be attacked selectively by a colloidal suspension of a fat-soluble mustard, say *H*, dissolved in a liquid fat.



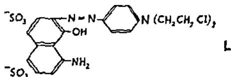
Another series of compounds which would be of great interest from the point of view of active transport are the azo compounds derived from *J*, now being studied at the Chester [illegible]

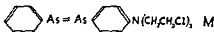
compounds to show a variable degree of active transport. For example, *J* itself would probably differ from *K*, and both





would probably differ from *L*, in manner of treatment by active transport mechanisms.





There are many other series of compounds which are worthy of study in *connection with active transport*, not least the arsenicals based on *M*.

The Nature of Adaptation and Resistance

When a drug is administered cellular adaptation may occur so that the drug is no longer effective. The adaptation may occur either in the host or in the tumour. For example, the development of an efficient hepatic detoxication mechanism, or of an efficient urinary excretion mechanism, may render a drug useless Adaptation in the tumour may take the form of a mass adaptation of tumour cells which may be permanent even in the absence of the drug and so confer permanent resistance. Or resistant tumours may arise by multiplication of occasional cells which by nature were resistant before application of the drug.

The mechanisms upon which adaptation and resistance are based are almost certainly variable. It is easy to see that resistance can arise in a tumour by (a) development of enzymic detoxication (an example of adaptive enzyme formation); (b) elimination of an activating enzyme, (c) development of an active transport mechanism which pumps the drug out of cells as fast as it penetrates. No doubt there are other physiological mechanisms which may be concerned.

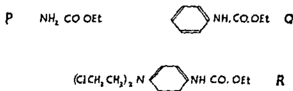
Mr. P. Hebborn and I have made a preliminary study of a Walker rat sarcoma resistant to compound *B*. This compound, as explained earlier, is believed to act after hydrolysis by a peptidase Resistance of this tumour to *B* does not arise in the host, for it continues when the resistant tumour is transplanted through several hosts. The resistance must therefore arise in the cells of the tumour. On the other hand, compared with a normal tumour the resistant tumour contains much less of the peptidase which is believed to activate *B* We are therefore considering the possibility that the resistance is *due to elimination of those tumour cells which normally* have a relatively high content of the activating peptidase.

The Exploitation of Adaptation and Resistance

The possibility exists that, if adaptation and resistance are based on physicochemical changes such as changes in enzymic constitution and in active transfer, then these

phenomena may be turned to advantage. The experimental evidence already available shows that not all of the tissues of the body become resistant, or at least not to the same degree. Thus when a prostatic tumour becomes resistant to œstrogen, the other tissues of the body are usually not resistant. When the Walker sarcoma becomes resistant to a nitrogen mustard, the general toxicity of the mustard remains unaltered, and the weight loss caused by a given course of mustard treatment is substantially unaltered. Thus resistance must frequently be limited to the tumour and perhaps a few other cell types in the body.

Where resistance is due to the formation of an adaptive enzyme, the principle which would underlie the use of adaptation is simple: when adaptative enzyme formation has reached the point where the drug in use is rendered ineffective by the detoxicating action of the drug, then this drug should be replaced by another which, instead of being detoxicated by the adaptative enzyme, is activated. For example, a tumour which becomes resistant to urethane *P* or ethylphenyl carbamate *Q* might be expected to do so by formation of an adaptive enzyme which will split either the ester or the peptide linkage. *P* or *Q* could then be replaced by a drug such as *R*, which should be activated by the adaptive enzyme.



Another method of approach would be to investigate which types of substances most readily cause formation of adaptive enzymes in tumour cells. Pretreatment of a tumour with such a substance could then be used to potentiate the action of a drug designed to be activated by the adaptive enzyme. For example, acetamide might act as a potentiator for *B*.

I am hoping in the next year or two to carry through a preliminary investigation of some of these possibilities.

Conclusion

It is evident from the discussion above that although our knowledge of cell biology is so restricted, even with present knowledge it is practicable to design a variety of programmes for deriving drugs of increased selectivity. In particular, it is necessary not to adopt a defeatist attitude when one encounters difficulties caused by the operation of biological variables such as adaptation and resistance If such phenomena are taken, not as mysterious limits to our scope but as processes involving tangible physicochemical mechanisms, such as enzymic phenomena, there is a good chance that what appear to be disadvantages may ultimately prove to be advantages.

But when this has been admitted, the fact remains that our knowledge of biological variables is extremely limited. The confines of constructive thought in the field of design of selective drugs are not set by the limitations of organic chemistry but by ignorance of cell biology. We need vastly more research on fundamental aspects of cell biology. While many methods must be used to obtain this fundamental knowledge, I venture to suggest that none will, in the long run, prove more profitable than those of enzymic cytochemistry (Pearse, 1953, Danielli, 1953)

I am indebted to the British Empire Cancer Campaign for a grant which has assisted this study

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DISCUSSION

HADDOW Prof Danielli might be interested to know we have already examined a large series of the azo compounds of the type he has

described and are still engaged on them now. We have also examined some of the urethane mustard types, one of which he mentioned, phenylurethane mustard (R)

DANIELLI· Have any of the azo compounds you studied sulphonic acid residues? I remember your results with the parent azo compounds were rather promising.

HADDOW: I think not.

TURNER· It appeared in your report that the free amino compound *A* was somewhat less active than the acetyl *B*. You attributed that, I suppose, to dosage?

DANIELLI: Yes. We gave one-third of the LD_{50} of each of these, which meant that we could give very much less of the free amino compound.

THORELL. What evidence do you have that the resistance is conditioned by the liberation of an active enzyme which is splitting the acetyl compound?

DANIELLI: Our evidence doesn't constitute proof that resistance is due to the enzymic factor, but it is an interesting coincidence that whereas there is a significant amount of the enzyme which will split off acetyl residue in the tumour before it becomes resistant, a resistant

GENERAL DISCUSSION

"Where do we go from here?"

ENG [illegible]
is fair [illegible]
taken [illegible]
of clas[illegible]
speakers We have heard about differences in myeloid and lymphoid [illegible]

[illegible] tween the different types of leukæmia. Also we have heard nothing about leukæmoid states, myeloid sclerosis, the so-called non-leukæmic splenomegaly and so on. I wonder whether the work with chemotherapeutic agents might help us there. Has any work been done on the treatment of the so-called leukæmoid reactions with the different substances we have heard about? Have some of these substances been tried in any of these obscure conditions, and can we learn anything about the relationship between what we call real leukæmias, in the classical sense of the word, and these perhaps related conditions?

Prof Witts mentioned yesterday the remarkable difference [illegible]

conditions

Another group of papers and discussions dealt with the regulation of [illegible]
I think [illegible]
especiall[illegible]
wonder [illegible]

[illegible] combine, for example, the shielded spleen experiments with tissue

culture work, using extracts or cells from different tissues after a procedure like that, and applying that to tissue culture of normal cells

A third and different approach to the whole problem was that of Dr. Gross, which I think must be looked into more deeply; we must try to repeat that in different laboratories. That too could perhaps be combined with other methods of attack. It is important that it should be tried with other strains and other combinations of strains, and I would like somebody to take that up, and study it in the light of what Dr. Lorenz said.

DE BRUYN: We still do not know what has gone wrong in the interaction between the leukæmic cells and their environment. In fact, we do not as yet know enough about the interaction between normal cells of the hæmatopoietic systems and their immediate environment. Therefore, more basic research still has to be carried out. Among the other lines of research, experimental cytology will probably play an important part. Observations on [illegible]

[illegible]

mediate environment, it is necessary to isolate the cells and tissues in artificial systems, in which the influence of the organism can be eliminated.

I know most of this work has had to be done by tissue culture techniques, and many workers have been discouraged by these rather elaborate and expensive methods. But during recent years some improvements in these techniques have been made, and at present we can cultivate normal and abnormal cells of the blood, and tissue fragments of the hæmatopoietic organs.

They can be maintained for a certain time under more or less controlled conditions and may be observed at will under the highest powers of the phase-contrast microscope. The advantage is that it is possible also to do experiments with human cells and human tissues. A great step forward would, of course, be the development of a culture medium of entirely known composition. [illegible]

[illegible] these strains has been derived from a transplantable mouse lymphosarcoma and has been maintained now for six years outside the

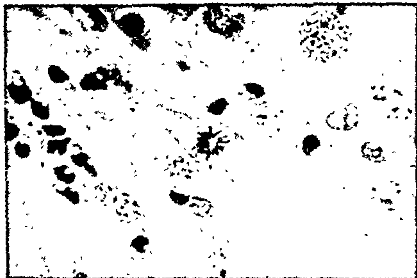


FIG 1. (de Bruyn) Fixed and stained culture derived from a transplantable lymphosarcoma of the mouse, designated as MB (T86157) The cells have been maintained in continuous culture for a period of more than five years obj 47 5, oc 15



FIG 2 (de Bruyn) Cells derived from a transplantable myeloid mouse leukæmia, designated as mIMl The cells have been maintained in continuous culture for a period of more than one and a half years Phase contrast Zeiss Winkel obj. 40, oc 9

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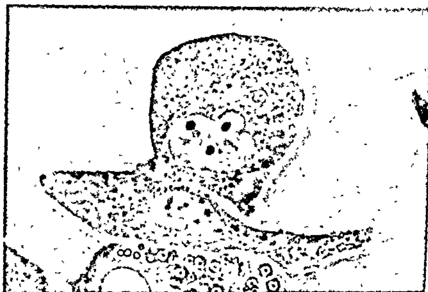


FIG. 3. (de Bruyn) Cells from the same culture Phase contrast
Zeiss Winkel, obj 100, oc 9

the organism, and therefore we continue to use these rather elaborate and expensive techniques.

Kieler: I should like to add a few words on the tissue culture question. I think that at the present moment we are facing an alternative; either we get unlimited growth without any cell differentiation, or we get some cell differentiation without any growth. What we want is both at the same time. I wonder

want so badly. I wonder whether Dr. de Bruyn has any further suggestions.

de Bruyn: I think it would be worthwhile trying to get the substance Dr. Jacobson talked about and testing this substance

organ as a whole or on separate cells in the tissue culture. Using the last method one has the advantage that the direct influence of these substances may be studied with the aid of the phase-contrast microscope.

Lorenz: There is agreement among radiologists that acute lymphatic leukæmia should not be treated with X-rays. We have a very acute lymphatic leukæmia in inbred guinea pigs which kills the animals in about three weeks, with a white count up to 900,000, a spleen weight up to 12 g, and dissemination into all organs and tissues. If you try to use the ordinary radiological

methods of small daily total body doses, the tumour just goes on; but if you give massive doses, as we can do now, keeping the animals alive by giving them bone marrow, you get some interesting effects.

Fig. 1 shows different treatments with high doses, 4×200 r every day, 5×200 r every day, and 5×400 r every day, and at

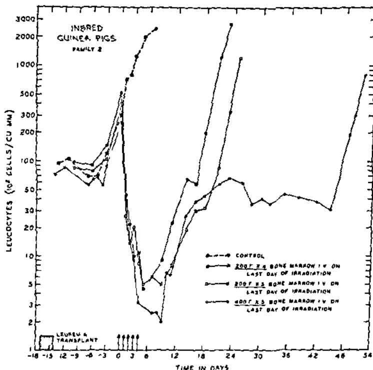


FIG 1 (Lorenz)

the end of the treatment, bone marrow injection. All *these* animals lived; but about half of those exposed to 2000 r died following treatment. The animals were irradiated when the leukæmia was well-established, with a count of about 50,000 With 2000 r the

In animals
e metastases
e irradiation
roscopically

only in the lymphatics, although the spleen went back to normal size. We also tried higher doses, but that is about the limit we can use—and it is quite remarkable that the guinea pig, with an LD_{50} of 400 r, can take 2,000 r and be kept alive.

If you plot time of remission against total dose, you get a straight line (Fig. 2) which probably means we could never cure acute lymphatic leukæmia with irradiation alone. We thought that as the tumour was very large in the spleen we could transplant

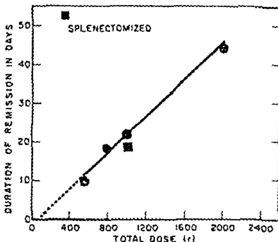


FIG 2 (Lorenz)

into splenectomized guinea pigs, but the effect of the irradiation is just the same.

Then we thought of combining X-rays and chemotherapeutic agents. We have only tried two so far, namely Aminopterin and TEPA (triethylenephosphoramide). Aminopterin in doses of 5 mg./per animal of approximately 500 g sometimes doesn't even touch the leukæmia. But if we give Aminopterin and X-rays simultaneously in a single dose, the time of remission is governed by the X-ray dose and not by the Aminopterin, although the effect on the leucocyte count is more pronounced than with either treatment alone. The picture is different however with TEPA. With an injection of 0 25 mg TEPA we get a remission of about

five to six days, and a second and third remission can be obtained, but then the animal dies, because the drug is too toxic. But if you combine TEPA and 500 r X-rays simultaneously (500 r gives a remission of about ten days), then we get a remission, in the few surviving animals, in excess of twenty days. This may mean that we have a synergistic effect. We want to try other drugs also. We could solve the problem perhaps if we had a drug that only attacks the lymphatics and not the bone marrow or other tissue

ISRAËLS: Perhaps as one of the original attackers of so many people here I may be allowed to make a few remarks. In the first place, you've got me all wrong! I am not against research on animal leukæmia. What I meant was that the work on animal *leukæmia has been conducted on too narrow a front. People have* been so fascinated by transmissible mouse leukæmia that all their energies have been canalized on to transmissible mouse leukæmia to the detriment of all other types. And it is the other types which are of more interest from the human side. What I want to see in the future is some attack on these dependent leukæmias that Dr. Furth talks about.

And I would like to see reopened the question of leukæmia in animals other than rodents Experience with anæmia shows how *misleading work with rodents can be. Surely you want to look* elsewhere. The pig is probably, from many points of view, the animal with a hæmopoietic system nearest to the human. Leukæmia was described in the pig many years ago—Dr. Engelbreth-Holm mentioned that in his book—but it has been left like that. The trouble is, I gather, that leukæmia in the pig only arises very infrequently. Now here we have been shown how you can produce leukæmia by irradiation. Admittedly that must be regarded as a somewhat crude technique, but if you could produce leukæmia in pigs by irradiation techniques, and you have whole

with them; and there are others.

It has also struck me that many workers use survival as a criterion of the effectiveness of drugs. In our present state of knowledge I am not sure that that is a very good thing to do. It isn't much good giving a certain drug and counting the dead bodies after a certain length of time. You want to know over

pattern, you find that something is happening. Mice only live three years, so
matter of hour
there is any cl

I wish very
for more systemization. Unless we understand ourselves and agree on nomenclature, we are going to get nowhere. May I suggest that we use the term "aleukæmic" only for conditions when the bone marrow is involved and the peripheral blood, as far as we can tell, is normal—we can't tell whether the lymphocytes we see are tumour cells or not at the moment—and keep the term "subleukæmic" for types with a low leucocyte count and some abnormal cells in the peripheral blood, with the bone marrow definitely involved, and don't use the term "leukæmia" at all for conditions in which neither the blood not the bone marrow is [illegible]

next generation, where are you going to end? Dr. Gross has an agent which produces salivary gland tumour or leukæmia indifferently. Are you going to call salivary gland carcinoma leukæmia? If we knew the ætiology we could call it the salivary gland tumour form of whatever disease it was, but we must draw the line somewhere when we don't know the ætiology.

With regard to non-leukæmic myelosis and so on, there is a lot of work to be done on the descriptive classification of clinical cases. One of the difficulties is to decide whether it is a primary disease or whether [illegible]

a lot of these cases with mustards, Myleran, cortisone and so on, and they are remarkably resistant to all these forms of treatment Those are the points I want to see followed up

GROSS I should like to correct several statements by Dr Israëls. He makes the criticism that we are fascinated by transmissible leukæmia and we should look at other forms of leukæmia. Some years ago mouse leukæmia was not transmissible from one strain to another, and Dr. Israëls would have been happy to use this type of leukæmia for his studies Then we found that if you use the newborn mouse you can transmit it very easily from one strain to another. So I don't think we can divide leukæmia into the

transmissible and non-transmissible. We may find that they are all transmissible provided the proper conditions are provided for transmission.

ISRAELS: That's quite possible; all I want is to have them studied.

GROSS: Dr. Israëls wonders whether we should draw a line at agents that cause both tumours and leukæmia. But I strongly suspect that I have at least two agents, which are hooked up, and one interferes with another. If we leave them alone only leukæmia develops, the leukæmia agent being the stronger one. But if we separate them under proper conditions we can get either disease. But this is just a digression. I wanted to say a few words about what I think of a future approach for fundamental research on the nature of leukæmia.

First, I think that the newborn animal should receive proper attention. And perhaps the newborn mouse is already too old; we should perhaps inoculate mice before they are born. What makes the newborn animal resistant a few hours after birth? That may be of some importance.

Another thing is that the AK strain gives agents which cause either leukæmia or salivary tumours—salivary tumours which very rarely develop spontaneously in mice. There may be many other strains with no incidence of tumours which might give us agents that cause different tumours in different strains. If you use a proper detector strain for the inoculation and a proper donor strain for provision of the extracts, you may find that quite a few of them carry tumour agents.

There are strains of mice which develop leukæmia in a certain percentage, and this can be increased by inbreeding, by certain

prop- may exist agents which are completely adapted to their hosts and will produce no symptoms unless activated. The problem of inducing agents should be studied.

Finally I would like to address myself also to the clinical group. Shouldn't we give much more attention to the possible familial incidence of leukæmia and tumours?

FURTH: The theme of this discussion is "Where do we go from here?" After these fertile discussions, most of us will go home

loaded with ideas beyond our capacity for investigation. Fortunately, our deliberations will be published. May I, therefore, comment further on a few problems which have come up.

[illegible]

exteriorized lymph-venous anastomosis should yield information on the circulation and rate of new formation of lymphocytes

[illegible]

but also in the bone marrow. There are experimental leukæmias

[illegible]

spleen and try to get out of it the hypothetical substance which causes leucopenia. One should also try to isolate it from normal and large spleens of different types and find out if it is a physiological substance.

I was also much impressed by Prof Haddow's talk about the possibility that the leukæmic cell may be due to deficiency of a certain enzyme and, perhaps, the leukæmic cell could be brought back to the normal type by "replacement" therapy This calls for much more elaborate studies of the biochemical processes of leukæmic cells versus normal cells, particularly of their enzyme systems Adequate controls are essential Excellent control

[illegible]

formed to show whether the cells actually did incorporate the enzyme.

The colchicine derivative Dr. Moeschlin talked about reminds me of a class experiment made many years ago. It was thought then that colchicine arrests cells in the metaphase and, if combined with irradiation, the latter would be much more beneficial since mitotic cells are much more sensitive to irradiation than normal

cells. The class experiment failed. The students did not check morphologically that colchicine as applied did arrest mitosis at the time the mice were irradiated. This type of experiment deserves further study.

We are, of course, all excited about Dr. Gross's work. I should like to see many more investigators studying the viral aspects of neoplasia; to what extent his work is applicable to different strains, species, types of leukæmias, and so on. There is a tremendous background of information in the studies of avian leukoses, rabbit papillomas and fibromas, and the milk factor of mice. I understand that the salivary gland tumour is not readily transplantable. If so, it might be worth while to search for antibodies and thereby demonstrate the virus by indirect means. I should like to hear suggestions as to how we could demonstrate the conditionality of hæmopoietic cells and assay hæmopoietic regulators.

MOESCHLIN: I should first like to stress some points about the problem of the two cell strains or two cell generations in leukæmia. First the acute leukæmia: I think the most essential point, as Dr. Engelbreth-Holm pointed out, is that these cells are not differentiated. Even if we treat them with Aminopterin, we sometimes see the normal cells come back again. Some authors think that these are matured cells, originating from the previously immature leukæmic cells, but I think there are many facts that demonstrate that this is not true. Many years ago we punctured leukæmia patients not only in the sternal marrow but also in the ribs, iliac crests, and so on. In one patient who was in a very early stage, I found myeloblasts in the sternal marrow. The same day and the day after I punctured different ribs and then the iliac crests on both sides, and I was astonished to see that he had only myeloblasts in one part of the sternal marrow. In about three months

all have, where myeloma was localised at the beginning only in

the first myeloma cells appeared in the sternal marrow, but not in the other bones; and later generalized in the whole marrow, ending in a plasma cell leukæmia. So I think there we have the whole evolution, as we have in acute leukæmia. If we treat these patients with some of these new drugs, there is no doubt that we knock out only the neoplastic forms of the cells, and the normal reticulum cells will start to produce normal cells again.

Perhaps I could just mention some points about chronic myeloid leukæmia. As a rule, if we can keep these patients alive they get a transformation to the acute myeloblastic form at the end. If you make different punctures of terminal cases at the same time (spleen, glands, and different parts of the bones), you may find cases which are just loaded with myeloblasts, for example in the ribs and not in the spleen, and some time later there will be metastases in other parts. So I think that in chronic myeloid leukæmia we have all the transitions from a relatively benign to a malignant tumour, as we have in fibromatosis evolving to fibrosarcoma.

A point which I think would be essential to study further is the different action of drugs on myeloid leukæmia. For example, X-rays and urethane: we have been very surprised that spleen punctures in urethane-treated patients demonstrated a much greater therapeutic effect on the spleen than on the sternal marrow, in contrast to arsenic, which acted on all the myeloid tissue (see Moeschlin, S., 1947, "Spleen puncture". London: Heineman) I think this point should be studied more intensively.

Then there is the puzzling problem of myelofibrosis. I think it is a syndrome. There are cases which transform at the end into an acute myeloblastic form, so in this type there is no doubt that they are true leukæmias Rohr thinks that in these forms the neoplastic transformation goes down to the reticulum cell, which can transform into fibroblasts, osteoblasts and even into leukæmic cells. Other forms which still show about 50-60 per cent lymphocytes in the spleen puncture may be due to a compensatory developing extramedullary myelopoiesis in the spleen by the obstruction of the bones. Here these drugs may be dangerous, as X-rays are known to be.

I think it is most essential for future research to try to get, as Dr de Bruyn pointed out, a leukæmic cell which we can maintain in cultivation As far as I know she has only succeeded with lymphosarcoma cells, and no one has kept myeloblastic cells longer than some months.

Since Dr. Gross can only infect the embryonic cell by the transfer of his agent, it may be that the embryonic cell and the neoplastic

cell have something in common in their metabolic processes. Many other things that have been found before to be typical of the neoplastic cell are found to be typical also for the embryonic cell. This is only a suggestion.

I would like to ask if there is a known strain of acute *myeloid* leukæmia which can be transferred by the cells from one animal to another. Those whom I have asked knew only of non-differentiated leukoblastic forms.

JACOBSON. We have been speaking of differential suppression of leukæmic tissue in order that the normal may flourish. This certainly occurs in cases of myelogenous leukæmia after splenic irradiation. In experimental animals, one can demonstrate differential suppression of growth of hæmatopoietic tissue. For example, after radiostrontium has been given to mice in a dose of 2 microcuries per gram, the bone marrow is destroyed. Erythropoiesis immediately becomes intensified in the spleen of these animals. In fact, the lymphatic cells may almost entirely [illegible]

even though the animals have not as yet become anæmic.

BURCHENAL: It seems to me the most important point that has been emphasized this week has been Dr. Law's point about the 8-azaguanine resistant strain of leukæmia, which somehow becomes more sensitive and can ultimately be cured in a certain percentage of cases with Amethopterin. Clinically I think that principle has considerable importance, although perhaps not with this particular drug Now with the latest techniques of isolating white cells and keeping them cold, one can do a lot of enzyme studies on the white cells isolated from a patient There is a problem in that many of the sensitive cases are of the subleukæmic type, where you do not have many cells to work with, but even now the techniques are being developed so that that type of leukæmia can be used for study. The difference between sensitive and resistant cells in composition or enzyme content or in the uptake of nucleic acid precursors would be of tremendous importance.

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Then combination therapy, as Dr. Law has mentioned, is of the greatest importance. It seems to me that in the treatment of

of the greatest importance here.

Thorell: We know too little about the normal processes governing growth and differentiation to be able to speak about specific disturbances. For example, it doesn't help you much to call a thing arrest of differentiation In hæmoglobin synthesis during erythropoiesis there are several steps: globin synthesis, porphyrin synthesis later on, and finally the incorporation of iron. Something affecting differentiation can affect any step of this

differentiation Every new technique of importance will give not only more details but also new aspects. I think that, as Prof

cell have something in common in their metabolic processes. Many other things that have been found before to be typical of the neoplastic cell are found to be typical also for the embryonic cell. This is only a suggestion.

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The use of these resistant lines of mouse leukæmia in screening has certain clinical applications, in trying to find drugs which might be beneficial in the clinical situations where the resistance had developed.

Then combination therapy, as Dr. Law has mentioned, is of the greatest importance. It seems to me that in the treatment of leukæmia you will never have a drug which is going to knock out all the leukæmic cells. The most you can expect is a slight selective suppression of the leukæmic cells. It may be that in a particular enzyme system or chain of enzyme systems you have several ... at each step ... the leukæmic ... e designing of ... of. Danielli is of the greatest importance here.

TURNER: I think I should call to your attention a new metabolic cycle which is undoubtedly of immense importance for hæmato-

THORELL: We know too little about the normal processes governing growth and differentiation to be able to speak about specific disturbances. For example, it doesn't help you much to

analyses; now one can measure different metabolic steps in the differentiation. Every new technique of importance will give not only more details but also new aspects. I think that, as Prof.

Engelbreth-Holm has already said, it is essential primarily to follow the normal processes.

FAGRAEUS: I wondered if it is not possible that in avian leukæmia and mouse leukæmia we are dealing with the same thing —now that Dr. Gross has shown that mouse leukæmia can be transmissible. I got the impression from what has been said here about the trial of drugs that you always use mouse leukæmia. Doesn't it work on fowl leukæmia?

Dr. Gross shows that we have to keep to very young mice to

animals are not completely developed and so they get impressed by the virus. It is a general finding in virus work that the susceptibility of animals is different to different viruses at different ages—usually greater the younger the animals are. With Coxsackie virus, for instance, you can only infect mice when they are less than three days old.

DARCEL: I am very interested in the possible relationship between virus and biochemical activity of erythroblastosis virus. Mommaerts *et al.* (1952, 1953)* claim to have demonstrated that virus concentrates from erythroblastosis plasma have high ATPase capacity. The partition of enzyme activity in their preparations closely paralleled the distribution of virus activity. I wondered whether Dr. Fagraeus and Dr. Thorell have done any work along these lines

FAGRAEUS: No, I have only recently heard of it.

DARCEL: Dr. Nils Lannek (personal communication) of the

FAGRAEUS: It gives us quite a lot to think about but we haven't gone any further.

diseases, it would be

*Mommaerts, E. B., Eckert, E. A., Beard, D., Sharp, D. G. and Beard, J. W. (1952). *Proc Soc exp Biol*, N. Y., 79, 450

Mommaerts, E. B., Eckert, E. A., Beard, D., Sharp, D. G. and Beard, J. W. (1953) *Proc Soc exp. Biol*, N.Y., 83, 479.

relationship between chemical constitution of a drug and its distribution in the human body. This information will not become available in a reasonable time unless a particular group of workers concentrates on this problem. Until this information is available it will be difficult to make the best use of chemical agents and isotopes.

cellular behaviour of these viruses.

* * * *

CHAIRMAN'S CLOSING REMARKS

throughout the discussion has been the interplay between these two points of view; the work on experimental leukæmia in animals perhaps representing the intellectual approach, as it were, and the work on patients representing the other side But there is clearly no antagonism between them, and one very obviously fertilizes the other. I think we

applicability to patients, may also be of very much more importance because they may be keys with which we unlock riddles of cell growth and differentiation.

The work of Dr. Gross is of course of extreme interest, and opens up very large territories for study. At first one might adopt almost a fatalistic view about it. Some years ago the late Prof. Ryle, that very great clinical teacher, was doing a ward round and he had an American student attending for some reason or other. He said to this student: "What do you know about leukæmia?" The student said. "Well, they do say there are two kinds, but they all die, so what the

capable of development.

Finally, it has been a great delight to all of us to renew some old friendships and to make so many new ones. I should like to say how immensely grateful we are to the Ciba Foundation for its stimulating and magnificent contribution to medical science and also how grateful we are to Dr. Wolstenholme and his staff for organizing this conference with such very great efficiency.

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